Sirtuin 1 and Angiotensin II as Inflammatory Modulators in the Development of Diabetes

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Abstract

Diabetes mellitus is a multifactorial metabolic disease characterized by elevated blood glucose due to pancreatic β-cell dysfunction and insulin resistance. The mechanisms of the initiation and progression of the disease are not fully understood but there is increasing evidence that inflammation plays a crucial effector role in the development of both type 1 and type 2 diabetes (T1D and T2D), leading to β-cell damage and β-cell death. Remaining β-cells compensate for the higher insulin demand until they fail. In T1D, additionally dysregulated immune tolerance along with autoantibodies against self-antigens leads to β-cell destruction. In this work, we are focusing on the role of two inflammatory modulators in the development of diabetes, angiotensin II (Ang II) and sirtuin 1 (SIRT1).

Ang II as central player of the renin-angiotensin-system (RAS) is classically known as regulator for local and systemic blood flow, body fluid homeostasis and electrolyte balance. However, after the discovery of a pancreatic local RAS, the connection between RAS blockage and the reduction of new onset diabetes has been found. In this work, we show that Ang II induces islet inflammation, β-cell dysfunction and β-cell death in rodent and human islets and in a mouse model of T2D, independently of vasoconstriction. Blockage of the master regulating pro-inflammatory cytokine interleukin-1β (IL-1β) by specific antibodies improved glucose tolerance and islet inflammation in Ang II-treated mice. This provides an additional rationale for the treatment of type 2 diabetes with anti-IL-1β antibodies.

The NAD^+ dependent histone and protein deacetylase SIRT1, the closest homolog to Sir2 in yeast, has attracted interest as a regulator of stress responses and longevity. The enzyme is implicated in various age-dependent diseases because of its potential to modulate cellular processes of metabolism and immune responses. In this work, we demonstrate an enhanced metabolic and islet activity along with reduced regulatory T-cells in a knock-in mouse model carrying a SIRT1 mutation, which is associated with familial autoimmune diabetes and colitis. Further, we show the beneficial influence of β-cell specific SIRT1 knock-out in the induction of T1D in mice. We hypothesize that context dependent overactivation by the mutated SIRT1 leads to enhanced insulin secretion, islet inflammation and an autoimmune-like phenotype along with reduced regulatory T-cells in our SIRT1 knock-in mice.

In turn, β-cell specific deletion of SIRT1 protects from the development of T1D, possibly by the induction of a “β-cell rest” and therefore islet recovery. Accordingly, SIRT1 inhibition and not activation in the context of T1D may have beneficial effects.

Altogether, immunomodulatory treatments by targeting inflammatory players such as Ang II or SIRT1 may have therapeutical value in the context of autoimmune diseases and diabetes.

List of Abbreviations

ACE	Angiotensin-converting enzyme
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
Ang II	Angiotensin II
AP-1	Activator protein-1
AROS	Active regulator of SIRT1
AT1R	Angiotensin receptor 1
AT2R	Angiotensin receptor 2
ATP	Adenosine triphosphate
CD	Cluster of differentiation
Cxcl1	Chemokine (C-x-c motif) ligand 1
DBC1	Deleted in breast cancer 1
DNA	Deoxyribonucleic acid
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraaceticacid
FAD	Flavin adenine dinucleotide
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FCS	Fetal calf serum
Foxo	Forkhead box class O
Foxp3	Forkhead box p3
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide 1
HDAC	Histone deacetylase
HFD	High-fat diet
HIF-1α	Hypoxia-induced factor-1α
IL-1β	Interleukin-1β
IL-1Ra	Interleukin-1 receptor antagonist
IFNy	Interferon y
LPS	Lipopolysaccharide

LXR	Liver X receptor
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
NAD^+	Nicotinamide dinucleotide
Nam	Nicotinamide
NAMPT	Nicotinamide phosphoribosyltransferase
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor- kappa B
NMN	Nicotinamide mononucleotide
NR	Nicotinamide riboside
OCR	Oxygen consumption rate
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGC-1α	Peroxisome proliferator activated receptor γ coactivator-1α
PPARα	Peroxisome proliferator activated receptor α
PPARγ	Peroxisome proliferator activated receptor γ
RORγt	RAR-related orphan receptor gamma t
ROS	Reactive oxygen species
SEM	Standard error of mean
Sir2	Silent information regulator 2
SIRT1	Sirtuin 1
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TGFβ	Transforming growth factor β
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Ucp2	Uncoupling protein 2
WAT	White adipose tissue

1 Overview

1.1 The endocrine pancreas

The body needs a system to rapidly and reliably react to changes in blood glucose levels. Specialized cells in the endocrine pancreas are responsible for the secretion of regulating hormones to ensure glycemic homeostasis. These cells are organized in clusters, so called islets of Langerhans. As shown in Figure 1, these islets of Langerhans are composed of three main cell types: glucagon secreting α-cells (in green), insulin producing β-cells (in red) and somatostatin containing δ-cells (in blue). This system is highly conserved in animals, even if there is wide species variability in islet structure and cell composition which might reflect evolutionary adaptations. In mouse islets, α- and δ-cells are organized in the periphery and insulin producing β-cells are found in the center of the islets (Fig. 1B). In humans, the islet architecture is generally less organized (Fig. 1A). Pancreatic islets are highly vascularized to enable a rapid distribution of the secreted hormones to the circulation. Additionally, immune cells, mainly macrophages, are found in the islets. After a glucose challenge, synthesis and secretion of the hormone insulin leads to a lowering of blood glucose levels by facilitating uptake and storage of glucose in peripheral tissues including fat, muscle and liver. Glucagon has antagonistic effects; it provides glucose to the circulation by inducing glycogenolysis and gluconeogenesis in the liver under low glucose conditions. In this work, we are focusing on the effects of insulin secreting β-cells, which act as important glucose sensors adjusting insulin secretion to the prevalent blood glucose levels.

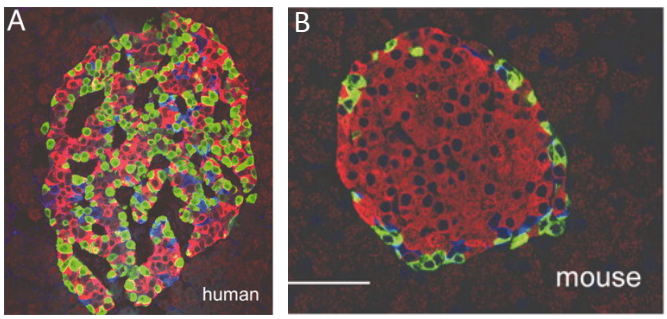

Figure 1. Fluorescent stainings of pancreatic islets in humans (A) and mice (B). Green = glucagon, red = insulin, blue = somatostatin. Adapted from [1].

1.1.1 Insulin secretion

In response to nutrient load, insulin is secreted by pancreatic β-cells in a glucose dependent mechanism (Fig. 2). Glucose enters the β-cell via facilitated diffusion through type 2 and type 1 (in human) glucose transporters (GLUT2/GLUT1) and glucose processing initiated by the enzyme glucokinase and via oxidative glycolysis leads to an elevation of the ratio of ATP/ADP. Thus, physiologically opened ATP-sensitive potassium channels close and induce the depolarization of the plasma membrane. Voltage dependent Ca^{2+} channels open and the increasing cytosolic concentration of free Ca^{2+} triggers exocytosis of insulin vesicles (Fig. 2).

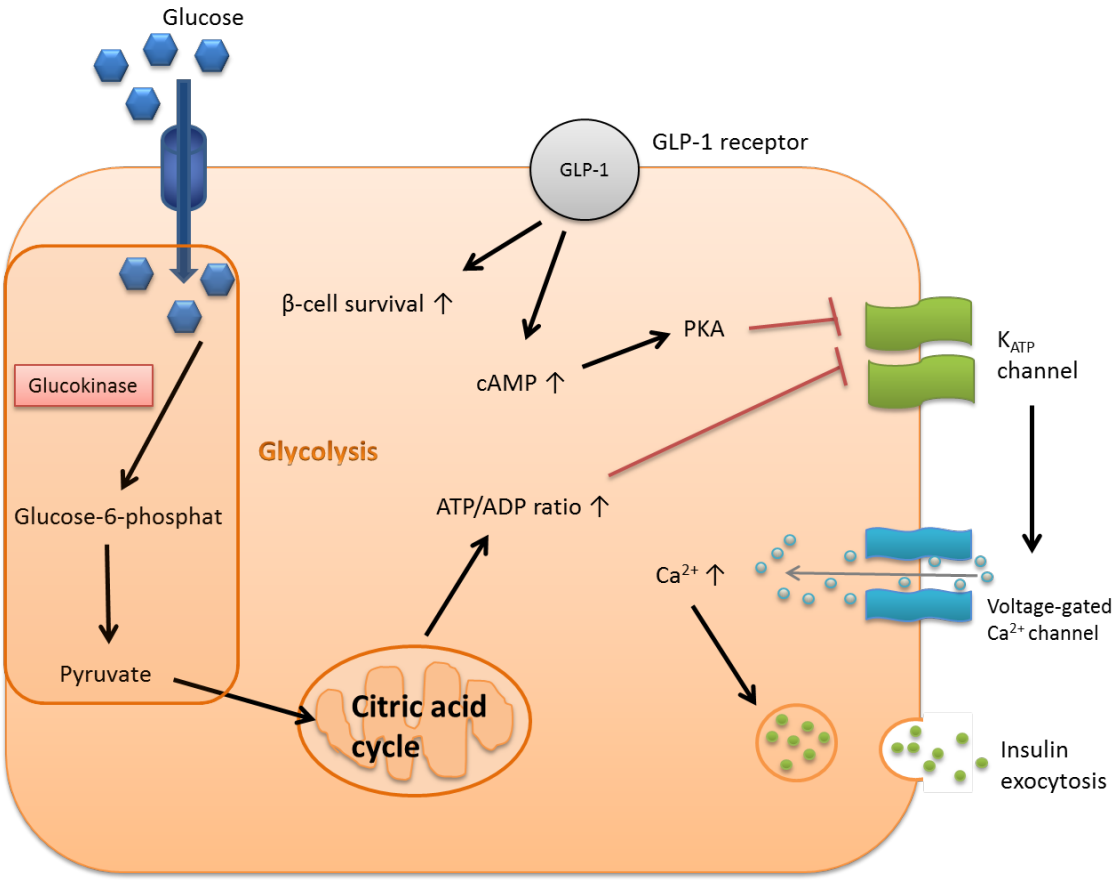

Figure 2. Glucose-stimulated insulin secretion in rodent pancreatic β-cells. Glucose enters the cell via type 2 glucose transporters (GLUT2) and is metabolized in different steps including glycolysis, initiated by the enzyme glucokinase, citric acid cycle and oxidative phosphorylation in the mitochondria. Thereby, ATP/ADP ratio increases, followed by closure of ATP-dependent K^+ channels. The cell membrane depolarizes and voltage-gated Ca^{2+} channels open causing Ca^{2+} influx and exocytosis of insulin vesicles. cAMP: cyclic adenosine monophosphate, PKA: protein kinase A. Adapted from [2].

The secretion of insulin is regulated by nutrients, glucose levels, hormonal and neural factors [3]. Gene expression levels of insulin are glucose dependently regulated via acetylation of histone H4 in the insulin promoter [4]. The glucagon-like peptide 1 (GLP-1) is one of the most important modulating hormones [5]. This incretin, which is mainly released by intestinal cells, is enhancing intracellular cyclic adenosine monophosphate and Ca^{2+} concentrations. Thereby insulin secretion and β-cell proliferation are stimulated and glucagon secretion is inhibited [6]. Additionally, modulators of the mitochondrial respiratory chain, such as uncoupling protein 2 (Ucp2), influence insulin secretion by uncoupling mitochondrial substrate oxidation from ADP phosphorylation [7]. Since the main function of β-cells is to sense glucose and directly translate into insulin secretion, they have low lactate-dehydrogenase levels leading to the production of mainly pyruvate and not lactate in the glycolytic cycle [8].

The glycolytic and respiratory metabolism is also coupled to the production of reactive oxygen species (ROS) in β-cells [9]. Oxidative stress is known to be an essential risk factor for β-cell dysfunction. It is defined as imbalance of ROS and antioxidative enzymes leading to the induction of genes involved in pro-inflammatory cytokine and chemokine production such as NF-kB. Oxidative stress disturbs the physiological function of DNA, proteins or lipids and impairs metabolism-secretion coupling [10]. Suppression of elevated ROS in a non-obese model of diabetes was shown to improve glucose-stimulated insulin secretion and ATP production by inhibiting lactate overproduction [11]. Since β-cells have low antioxidative capacities they are more susceptible to oxidative stress damage than other cell types [12]. However, growing evidence indicates also messenger function of ROS, especially H_2O_2, in the context of insulin secretion [13], [14], [15].

1.2 Principles of inflammation and immunity

The principles of immunity are based on two major subsystems, the innate and the adaptive immune system. Acute inflammation is classically known as the initial defense mechanism orchestrated by the innate immune system against bacterial infections and tissue damage [16]. It evolved as a physiological adaptive response to restore homeostasis but it may lead to chronic inflammation if unresolved.

The recruitment of leukocytes, specifically macrophages and neutrophils, leads to a classical non-specific inflammatory reaction including engulfment of pathogens, cytokine and chemokine release and transport of plasma proteins to the affected tissue. This response is triggered by receptors of the innate immune system, including toll-like receptors (TLRs) and cytokine release upon activation, such as IL-1, IL-6, IL-10 and TNFα. Several chemokines are involved in the recruitment and activation of innate immune cells at the site of inflammation, such as monocyte chemoattractant protein-1 (MCP-1) and chemokine (C-x-c motif) ligand 1 (Cxcl1).

Interestingly, inflammation can also be induced in the absence of any exogenous microorganism but in response to tissue malfunction or stress. This sterile inflammation is suggested to contribute to chronic inflammatory diseases [17] and it is likely to happen also in obesity, induced by metabolic stress.

T-lymphocytes, special cells of the adaptive immune system characterized by the expression of CD3, are able to react specifically to pathogens in an antigen-dependent manner and provide long-time immunity. Antigen-presenting cells (APCs) including monocytes, dendritic cells and B-cells induce the activation and clonal expansion of naïve lymphocytes in peripheral lymphoid organs by interacting through an immunological synapse (Fig. 3).

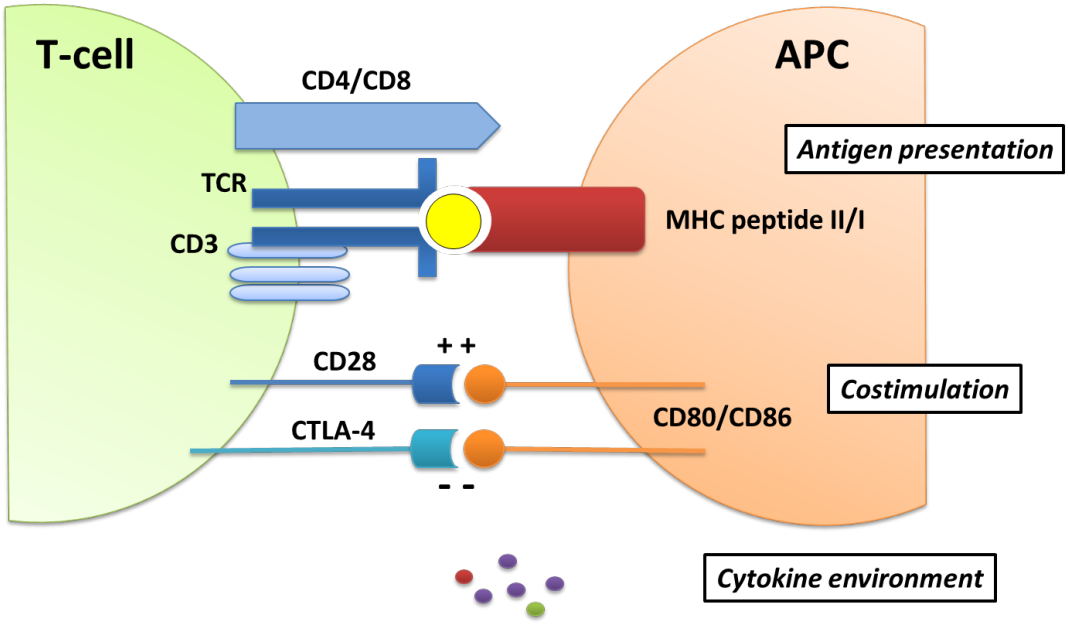

Figure 3. T-cell antigen recognition in the immunological synapse. T-cell activation and differentiation is induced via antigen presentation by antigen-presenting cells (APCs) and costimulation via receptors and the cytokine environment. TCR: T-cell receptor. CTLA-4: cytotoxic T-lymphocyte associated protein-4. MHC: major histocompatibility complex. Adapted from [18].

Mainly two types of effector T-cells are discriminated: $CD8^+$ cytotoxic T-cells and $CD4^+$ helper T-cells. Cytotoxic T-cells recognize antigens presented by major histocompatibility complex class I (MHC I) molecules leading to direct killing of infected cells via production of cytokines and perforins. Naïve CD4 T-lymphocytes can differentiate in distinct subpopulations of T-cells after recognition of antigens presented via MHC II molecules. Each lymphocyte carries a distinct antigen receptor and the ones binding ubiquitous self-antigens have to be eliminated during development to ensure self-tolerance. If costimulation via CD28 as the second signal for activation is missing during the first antigen exposure (Fig. 3), T-cells become anergic and do not respond to subsequent stimulation, ensuring self-tolerance. Depending on costimulatory signals, $CD4^+$ helper T-cells can differentiate into four different subsets with distinct immune properties: Th1, Th2, Tregs and Th17 cells.

Each T helper subset is defined by specific cytokines required for polarization, transcription factor programming and in turn, cytokines that are secreted, as illustrated in Figure 4.

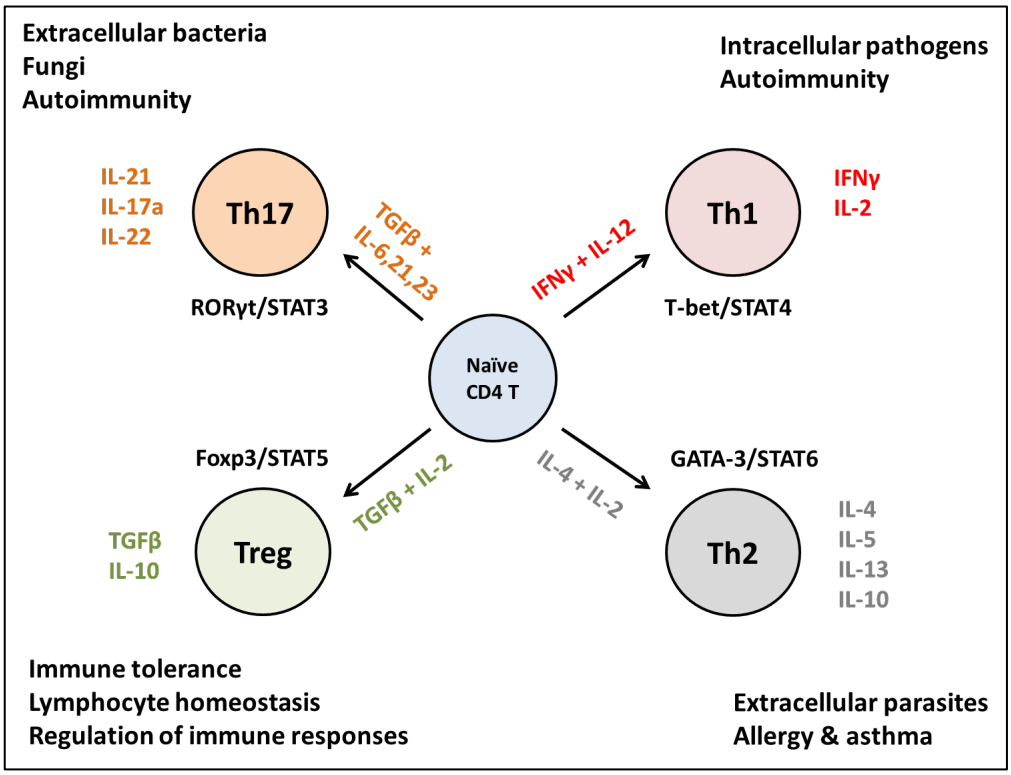

Figure 4. Model of naïve CD4 T-cell differentiation into major T-cell subsets with characteristic transcriptions factors (black), cytokines for their fate determination (colored) and unique products (colored, alongside). Adapted from [19].

Activated Th1 and Th17 cells are mainly involved in bacterial infections and autoimmunity, whereas Th2 cells are responsible for allergic inflammatory diseases and protection against parasitism. The differentiation into Th1 cells activates macrophages and cytotoxic T-cells and promotes CD4 T-cell memory. The production of cytokines by Th2 cells stimulates B-cells, eosinophils and mast cells and can also suppress Th1 immune responses.

Of particular interest, regulatory T-cells (Tregs) are specialized cells, which exert cell extrinsic immunosuppression [20]. The transcription factor Foxp3 is required for the development of Tregs [21] and its continuous expression is crucial for the maintenance of self-tolerance and tissue homeostasis [22], [23], as Scurfy mice with loss-of-function mutations in the *Foxp3* gene show multiple autoimmune manifestations [24].

There are mainly four mechanisms used by Tregs to suppress immune responses: direct modulation of APC function by suppressing the costimulatory receptors CD80/CD86 through molecules such as cytotoxic T-lymphocyte associated protein-4 (CTLA-4), killing of target cells via granzymes and perforins, disruption of metabolic pathways through degradation of ATP and the production of anti-inflammatory cytokines, such as TGFβ, IL-10 and IL-35. Tregs express the highest levels of CD25 (IL-2 receptor α), which suggests the importance of interleukin 2 for their suppressive function, although all activated T-cells express CD25. Defective numbers or functions of Tregs were linked to several autoimmune diseases including T1D [25], [26], [27], although there are discrepancies in the literature.

Interestingly, IL-22, which is classically involved in Th17 cell-induced inflammation, was shown to reverse obesity-induced metabolic disturbances and pancreatitis [28], [29]. Of note, the highest levels of IL-22 receptor 1 expression are found in the pancreas, including islet cells [30].

Beside the distinct differentiation factors for each T-cell subset, T-cell activation requires the involvement of other transcription factors, including AP-1, NF-κB, and nuclear factor of activated T-cells (NFAT).

Antigens presented in the periphery can also bind directly to B-cells, which are characterized by the expression of CD19 and B220, and a second signal for differentiation into antigen-producing plasma cells is delivered by activated T-cells. After infection defense, memory B- and T-cells are generated to ensure immunological memory in response to a second exposure to the antigen.

1.3 Diabetes and Inflammation

Diabetes mellitus is a chronic metabolic disease characterized by elevated levels of blood glucose. It is a major cause of morbidity and mortality worldwide. 90% of diabetic patients have type 2 diabetes (T2D). The development of the metabolic disorder has underlying genetic factors and environmental causes, including overnutrition and a sedentary lifestyle. Typically, the development of T2D is associated with insulin resistance in the insulin target tissues while β-cells compensate for the higher insulin demand. Failure of β-cell adaptation results in hyperglycemia and β-cell destruction [31], [32]. However, many insulin-resistant subjects never become diabetic because their β-cells can adjust their insulin production [33]. In the last years it became increasingly evident that a pathological involvement of the immune system plays a major role in the development of T2D (Fig. 5). Overnutrition causes a chronic pro-inflammatory state in many organs including the pancreatic islets [34], [35], [36], [37]. Inflammatory cytokines, immune cell infiltrations, apoptosis and fibrosis are elevated in islets of T2D animal models and in T2D patients [38], [39], [40], [41], [42].

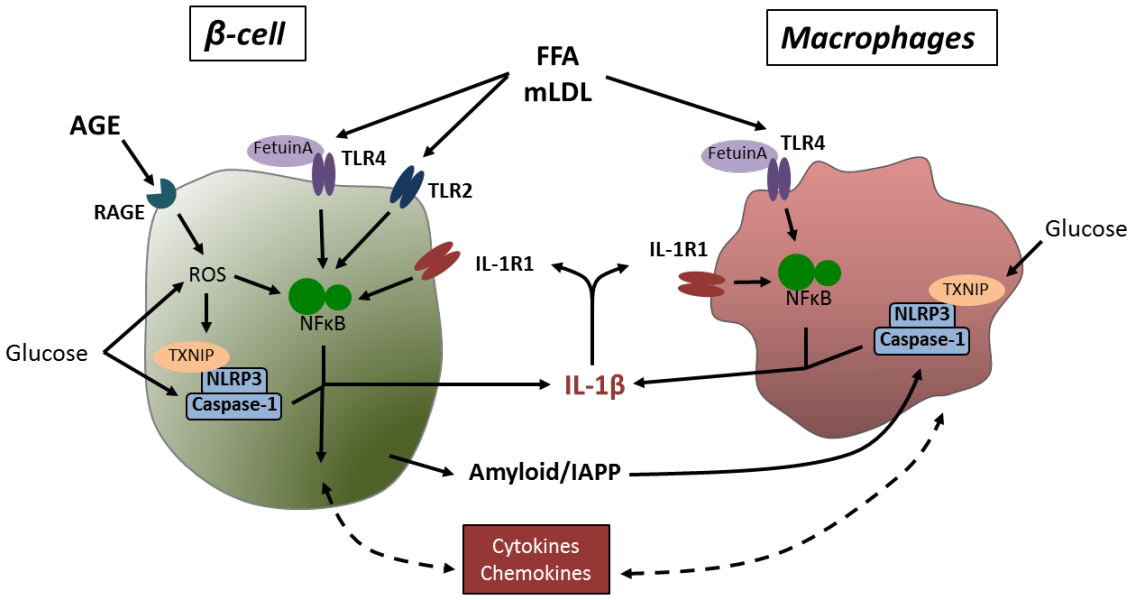

Figure 5. Model of inflammation in response to metabolic stress in pancreatic islets. Increased circulating free fatty acids (FFA), modified LDL (mLDL) particles and advanced glycation end products (AGE) bind to their cognate receptors leading to NF-κB activation and the production of various proinflammatory chemokines and cytokines, including the proform of IL-1β. Glucose-activated caspase-1 inflammasome causes overproduction of IL-1β in both β-cells and macrophages, which in turn triggers an autoinflammatory chronic response in the islets. Adapted from [34].

Persistently elevated levels of glucose and free fatty acid (FFA) lead to chronically increased concentrations of Interleukin-1β (IL-1β), which is one of the master regulating pro-inflammatory cytokines [35], [43]. Glucose-activated caspase-1 inflammasome causes overproduction of IL-1β, which in turn triggers an autoinflammatory process [43], [44]. The emerging imbalance of IL-1β and its naturally occurring antagonist interleukin-1 receptor antagonist (IL-1Ra) was shown to be involved in islet inflammation [39]. IL-1β producing macrophages are activated and contribute to the development of insulitis [41], [38]. Inhibitors of IL-1β such as anakinra, the recombinant form of IL1-Ra, or antibodies against IL-1β were shown to improve hyperglycemia, β-cell function and systemic inflammation in T2D and obese patients [45], [46], [47], [48], [49], [50], [51]. Hence, these blockers are in clinical development for the treatment of diabetes.

Type 1 diabetes (T1D) is a heterogeneous chronic disease characterized by T-cell-mediated autoimmune reactions to pancreas-specific antigens leading to destruction of pancreatic β-cells and insulin deficiency. Autoantibodies are present in 85-90% of T1D patients, who mostly become dependent on insulin treatment. The multifactorial disease is influenced by genetic and environmental factors such as viruses and toxins. It is thought to begin when unexplainable β-cell death leads to the exposure of β-cell antigens followed by T-cell activation through antigen-presenting cells. Further, there is emerging evidence that regulatory T-cells (Tregs) are less functional in suppressing effector T-cells leading to failure of maintaining self-tolerance in T1D [52]. However, the mechanisms involved in the destruction of β-cells and how they can be targeted is not fully understood. The development of combination immunotherapies with drugs that stimulate β-cell regeneration are promising and raise hope for finding a cure for T1D [53]. Besides the non-obese diabetic (NOD) mouse, a commonly used model for T1D in animals is the multiple low dose streptozotocin (mldSTZ) model leading to pancreatic insulitis and β-cell destruction [54]. STZ has structural similarity to glucose and enters the β-cell via GLUT2. It activates the DNA repair enzyme poly (ADP-ribose) polymerase (PARP), which consumes the coenzyme nicotinamide dinucleotide (NAD) leading to β-cell death.

1.4 The renin-angiotensin system (RAS)

The renin-angiotensin system (RAS) is originally known to play a crucial role in the regulation of local and systemic blood flow, body fluid homeostasis and electrolyte balance. The system consists of several components in various tissues, which act together in a complex enzymatic setup [55]. Renin, a kidney-derived protease, is released by the juxtaglomerular apparatus to cleave the hepatic precursor angiotensinogen. Subsequently, the inactive product angiotensin I (Ang I) is hydrolyzed by the angiotensin-converting-enzyme (ACE) to form the octapeptide angiotensin II (Ang II) that is highly active and the major effector of the RAS. Ang II has several receptors but most of the known effects are mediated by two opposing G protein-coupled receptors: Ang II type 1 receptor (AT1R) and Ang II type 2 receptor (AT2R) [56], [57]. Binding of Ang II to the AT1R triggers vasoconstriction and the release of aldosterone. The steroid hormone aldosterone is increasing sodium and water retention by binding to the mineralocorticoid receptor and thereby increasing also blood pressure and blood volume. However, there are many more known biological actions of Ang II including the induction of ROS, fibrosis and proliferation. Additionally, there are other angiotensin peptides including Ang III, Ang IV and the vasodilative angiotensin 1-7 (Ang 1-7), which is formed by the angiotensin-converting enzyme 2 (ACE 2) opposing the signal of Ang II [58], [59]. There are clinically used blockers of the RAS, which are either inhibiting AT1R (names are ending on “-sartan”) or inhibiting ACE (names are ending on “-pril”).

1.4.1 Local tissue RAS

In the past few years, the existence of functional local RAS in various tissues including the brain, kidney, heart, liver, skin, vasculature, immune cells, adipose tissue and the pancreas [60], [61], [62], [63], [64], [65] has become increasingly clear. All of the local RAS components are regulated within local tissues [66]. Abadir et al. even identified a functional mitochondrial angiotensin system [67]. These discoveries have expanded the understanding of RAS signaling and invited to new definitions for the RAS system beyond being a mediator of vasoconstriction.

1.4.2 Local pancreatic RAS

In 1991, Chappell et al. discovered an intrinsic angiotensin system in the canine pancreas [68]. Further components of the RAS were also found in the pancreatic islets of rodents and humans. Ang II was immunohistochemically localized in the mouse pancreas by Leung et al. in 1998 [69] and a tissue RAS was discovered also in human islets by Tahmasebi et al. [70]. Furthermore, the expression of ACE and AT1R was shown in islets by immunohistochemistry and quantitative PCR [71]. AT1R have been reported to co-localize with β-cells in the islet core, whereas AT2R mostly are found in the outer islet areas co-localizing with δ-cells. It was proposed that this local pancreatic RAS system contributes to the regulation of islet structure and function (Fig. 6).

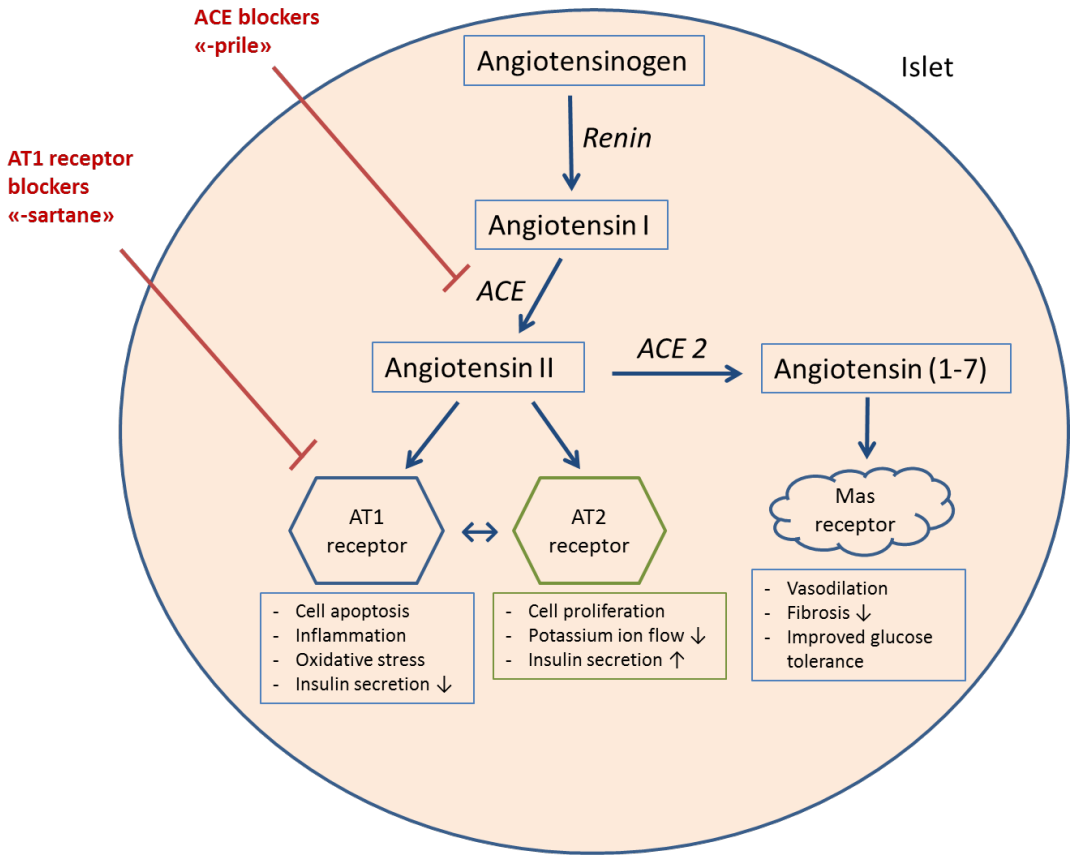

Figure 6. **Local renin-angiotensin system (RAS) in pancreatic islets**. Angiotensinogen is produced mainly in the areas of α-cells, it is cleaved by renin to yield angiotensin I which is processed by ACE to obtain angiotensin II. Acting via two receptors, angiotensin 1 receptors (co-localizing with β-cells) and angiotensin 2 receptors (co-localizing with δ-cells), angiotensin II is influencing metabolic homeostasis by modulating insulin secretion and inflammation. The enzyme ACE 2 is transforming angiotensin II to angiotensin (1-7), which is opposing the effects of angiotensin II via the Mas receptor. Inhibitors of the RAS, including angiotensin 1 receptor blockers ("-sartane") and angiotensin converting enzyme blockers ("-prile"), are clinically used. Adapted from [72].

Several major islet RAS components are elevated under hyperglycemic conditions, even independently of the presence of hypertension [73], [74], [75].

1.4.3 RAS and the metabolic syndrome

In addition to the discovery of a local pancreatic RAS, a connection between RAS blockage and improvement of metabolic diseases has been found. Hypertensive patients have a 3.3-fold higher risk to develop diabetes [76]. Vice versa, the metabolic syndrome is associated with hypertension and activation of the RAS [77]. Blocking of this RAS activation for anti-hypertensive treatment also reduced the incidence of metabolic disorders. Therefore, RAS inhibition has been shown in a variety of clinical trials to delay new-onset of T2D [78], [79], [80], [81], [82]. Searching for explanations, Ang II was found to inhibit glucose-stimulated insulin secretion [83], [84], to decrease islet blood flow [85], [86], [87] and to drive islet fibrosis. Furthermore, Ang II can induce insulin resistance [88], [89] by decreasing blood perfusion in insulin target tissues [90] and affecting skeletal muscle insulin signaling [91]. Several groups demonstrated the improvement of islet function, glucose tolerance and other diet-induced damages in animal models of T2D after RAS blockage [74], [92], [93], [94], [95], [71], [96]. Inhibition of the RAS in isolated islets of diabetic mice revealed direct effects of Ang II on islets. This includes improvements of insulin secretion and a lowering of elevated glucagon release [97], [84], [73].

However, the exact mechanisms of RAS blockage *in vivo* are only partly understood and it is not clear whether normalization of the blood pressure or direct inhibition of local RAS leads to the observed improvements of metabolic parameters.

1.4.4 Angiotensin II and inflammation

Although Ang II was classically known to regulate circulatory homeostasis, focus has recently turned to Ang II as a powerful pro-inflammatory mediator [98], [99], [100]. Activated RAS induces oxidative stress by stimulation of the NADPH oxidase [101] and may thereby trigger inflammation. Ang II plays a role in inflammatory processes in the kidney [102] and induces MCP-1 in pancreatic cancer cells [103] and the β-cell line RIN [104]. Ang II is involved in the recruitment of macrophages in atherogenesis [105] and in the arterial expression of the chemokines IL-8 and MCP-1 [106]. In blood mononuclear cells, AT1R antagonists and ACE inhibitors decrease the production of tumor necrosis factor (TNF), IL-6 and IL-1, independent of antihypertensive effects [107], whereas the anti-inflammatory cytokine IL-1Ra is increased [108]. In RAW 264.7 cells and various other cell types, Ang II induces the production of cytokines and the transcription factors NF-kB and AP-1 [109], [110], [111], [112]. Additionally, clinical trials revealed anti-inflammatory effects of RAS blockage, indicated by reduced levels of the proinflammatory markers TNFα, IL-6 and C-reactive protein [113], [114], as well as MCP-1 in the circulation of patients with cardiovascular diseases [115]. Similarly, blockage of the RAS in high-fat diet or streptozotocin (STZ)-induced diabetic rodents lowers MCP-1 and interferon y (IFNy) levels in the circulation and MCP-1 mRNA expression in the kidney [116], [94]. In pancreatic islets of high-fat diet fed mice treated with RAS blockers, proinflammatory genes are downregulated [94], [93].

1.5 Sirtuins

The silent information regulator 2 (*Sir2*) gene was originally discovered in 1979 as an enzymatic chromatin regulator in the yeast strain *Saccharomyces cerevisiae* [117]. It was found to increase yeast replicative lifespan and to slow down aging by supporting caloric restriction-induced processes [118], [119]. Since principles of energy metabolism are similar between species, structural and functional Sir2 homologs were also found in bacteria, plants and mammals, the so-called sirtuins [120]. Sirtuins are a conserved family of NAD^+ dependent cellular stress sensors, which affect a wide range of biological pathways related to aging, glucose and lipid metabolism, circadian rhythm and inflammation [121]. They couple metabolism with longevity since they induce the adaptation of numerous cellular activities during stress and aging such as cell cycle control, differentiation, microtubule organization and repair of DNA breaks [122].

The molecules are class III histone deacetylases (HDACs) that remove acetyl groups from histones to facilitate compact chromatin structures and gene silencing, thereby counteracting the effects of histone acetyltransferases. Despite their name, HDACs can also deacetylate non-histone proteins. There are other classes of HDACs, class I and class II, which regulate transcriptional activity but have no sequence similarity to sirtuins [123].

Seven different mammalian sirtuins (SIRT1-7) with different localizations and functions are known. They are activated tissue dependently to mainly regulate metabolic homeostasis during low energy availability, when the NAD^+/NADH ratio rises, or through cellular stressors. A conserved catalytic core that is able to bind NAD^+ and acetyl-lysine substrate is shared by all sirtuins. They act as deacetylases, ADP-ribosyltransferases and have other deacylase activites to modify the expression of target genes or directly interact with proteins. In their function as protein deacetylases, sirtuins transfer acetyl groups from lysine residues of target proteins to ADP-ribose (Fig. 7), leading to the generation of the deacetylated end product, nicotinamide (Nam) and O-acetyl-ADP-ribose.

SIRT1 and 6 are mainly nuclear sirtuins, whereas SIRT2 is found in the cytoplasm, SIRT3, 4 and 5 in the mitochondria and SIRT7 in the nucleolus [124]. SIRT2 was shown to regulate cell cycle control and microtubule organization [125], [126] and SIRT6 has been implicated with caloric restriction and genome protection [127]. SIRT3 is the most studied member of the

mitochondrial sirtuins, which are suggested to regulate mitochondrial physiology, glucose and lipid metabolism. SIRT3 supports mitochondrial oxidation by suppressing glycolysis [128], [129], [130], induces insulin sensitivity and is involved in the regulation and protection of pancreatic β-cells in mice and T2D patients [131]. Contrary, SIRT4 blunts insulin secretion by inhibition of glutamate dehydrogenase and suppresses fatty acid oxidation [132]. SIRT5 was reported to detoxify excess ammonia by controlling the urea cycle. SIRT6 and SIRT7 are suggested to be involved in aging and tumorigenesis [133]. However, little is known about the enzymatic activities and functions of SIRT 5, 6 and 7.

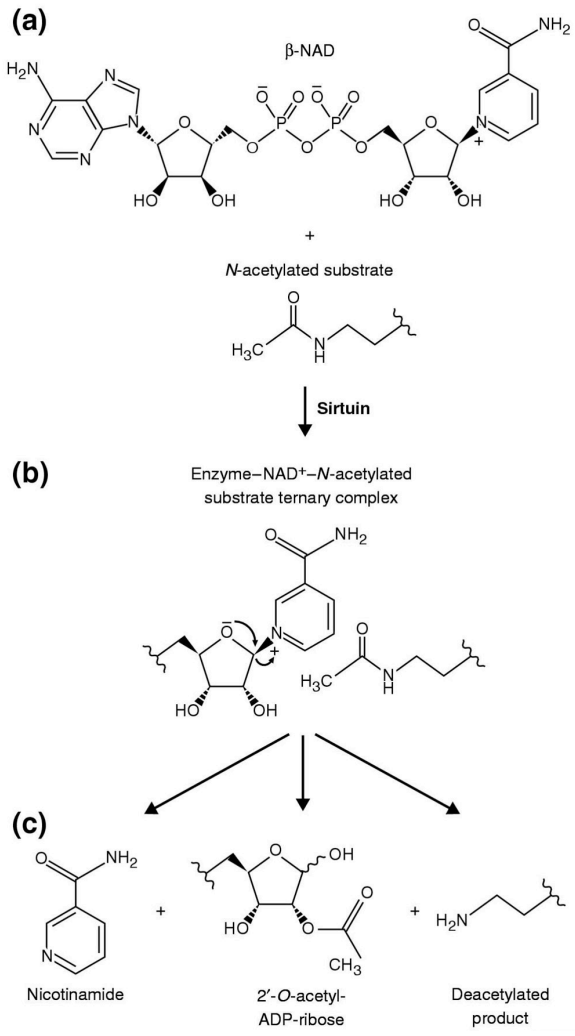

Figure 7. The enzymatic deacetylation activity of sirtuins. (a) Nicotinamide dinucleotide (NAD) acts as cofactor in deacetylation processes of sirtuins. (b) An intermediate enzyme-NAD-acetylated substrate complex is built and results in (c) nicotinamide, O-acetyl-ADP-ribose and the deacetylated product. Taken from [134].

1.5.1 NAD^+

In mitochondria, energy is stored in carrier molecules such as reduced nicotinamide dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$), which can be oxidized to nicotinamide dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD^+) to generate ATP in the mitochondrial electron transport chain. The carrier molecules act as electron donor and the generated energy is used to create a proton gradient that drives ATP synthesis.

When carbohydrates or fatty acids are metabolized in catabolic cellular processes, NAD^+ is reduced to NADH. By contrast, when energy levels in the cell are low, NAD^+ levels rise. Furthermore, NAD^+ acts as cofactor for many dehydrogenases and deacetylases, including sirtuins, in various metabolic processes. When NAD^+ levels increase, indicating starving or stress conditions like exercise, sirtuins are activated tissue dependently to regulate energy supply [135], [136], [137]. Thus, NAD^+ is an important sensor and modulator of bioenergetic states. In its function as cofactor for SIRT1 and because rising NAD^+ levels activate SIRT1, precursors of NAD^+ are used as SIRT1 activators.

NAD^+ can be synthesized de novo or by recycling from nicotinamide (Nam), which is generated in NAD^+ dependent reactions (Fig. 8) and may act as feedback inhibitor of NAD^+ and SIRT1 [138]. The rate-limiting enzyme in the NAD^+ biosynthesis is nicotinamide phosphoribosyltransferase (NAMPT) that induces the conversion of Nam to nicotinamide mononucleotide (NMN), which in turn is converted to NAD^+ by the NMN adenylyltransferase (NMNAT) [139]. The vitamin nicotinamide ribose (NR) is a natural precursor of NAD^+, which can be converted to NMN by the nicotinamide riboside kinase (NRK). NAD^+ biosynthesis was impaired in obese, diabetic and aged animal models and diet- and inflammation-induced islet dysfunction was restored by NR or NMN substitution [140], [141], [142].

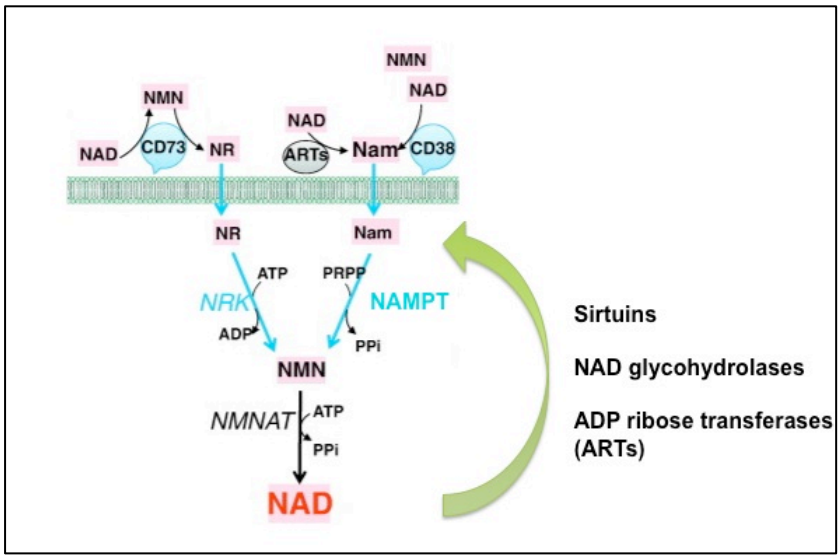

Figure 8. Recycling route of NAD biosynthesis in mammals. NAD is used as cofactor by sirtuins and other enzymes leading to the release of Nam. In recycling steps, Nam is converted to NMN by NAMPT and NMN is converted back to NAD by NMNAT. The NMN pool can further be filled by NR that is converted to NMN via NRK. NAD: nicotinamide dinucleotide, NMN: nicotinamide mononucleotide, Nam: nicotinamide, NR: nicotinamide riboside, NAMPT: nicotinamide phosphoribosyltransferase. NMNAT: NMN adenylyltransferase, NRK: nicotinamide riboside kinase, PRPP: phospho-ribosyl pyrophosphate. Adapted from [143].

1.5.2 Sirtuin 1

Sirtuin 1 (SIRT1) is an NAD^+ dependent class III histone and protein deacetylase, which belongs to the highly conserved sirtuin family and has tissue specifically various functions. It is the most studied sirtuin since it has attracted a lot of interest as the first identified mammalian homolog of Sir2, which was shown to influence longevity in yeast [144]. In adult tissues, SIRT1 is expressed ubiquitously; several studies showed its expression in liver, brain, adipose tissue, lung, spleen, muscle, thymus, pancreas and less in heart and the kidney. The highest expression levels were found in immune cells, especially B-cells, and the uterus (Fig. 9).

The human SIRT1 protein consists of 747 amino acids and has a molecular weight of 81.7 kDa. It is mainly located in the nucleus where it acts as transcriptional repressor via histone deacetylation [145] but also many cytosolic functions are known [124]. The subcellular localization varies depending on the type of tissue, stress levels and molecular interactions.

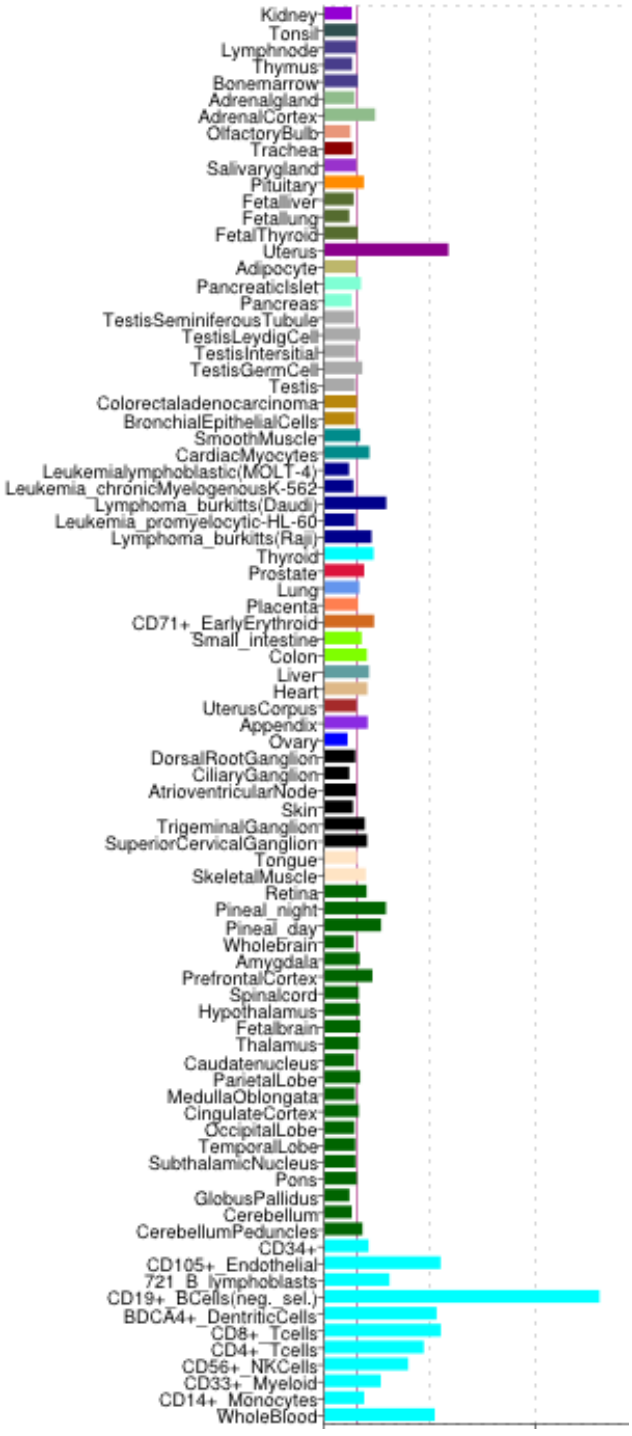

Figure 9. Relative mRNA expression levels of *Sirt1* in humans. *Sirt1* is expressed ubiquitously in humans but highest mRNA expression levels are found in the uterus and in blood cells such as B-cells. Taken from www.biogps.org

The functions of SIRT1 are manifold; it mainly plays a role in adaptation to stress responses by modulating metabolic homeostasis, circadian clock, immunity and more general epigenetics. SIRT1 has been linked to several age-related diseases including cancer [146], Alzheimer's disease and atherosclerosis.

There are more than 80 known protein substrates (Fig. 10) including forkhead box class O (Foxo), peroxisome proliferator activated receptor γ (PPARγ), liver X receptor (LXR), peroxisome proliferator activated receptor γ coactivator 1α (PGC-1α), uncoupling protein 2

(Ucp2), forkhead box P3 (Foxp3), nuclear factor-kappa B (NF-κB), hypoxia-induced factor 1α (HIF-1α), c-Jun and c-Myc that are components of the transcription factor complex activator protein-1 (AP-1), and the tumor suppressor p53 [147], [148].

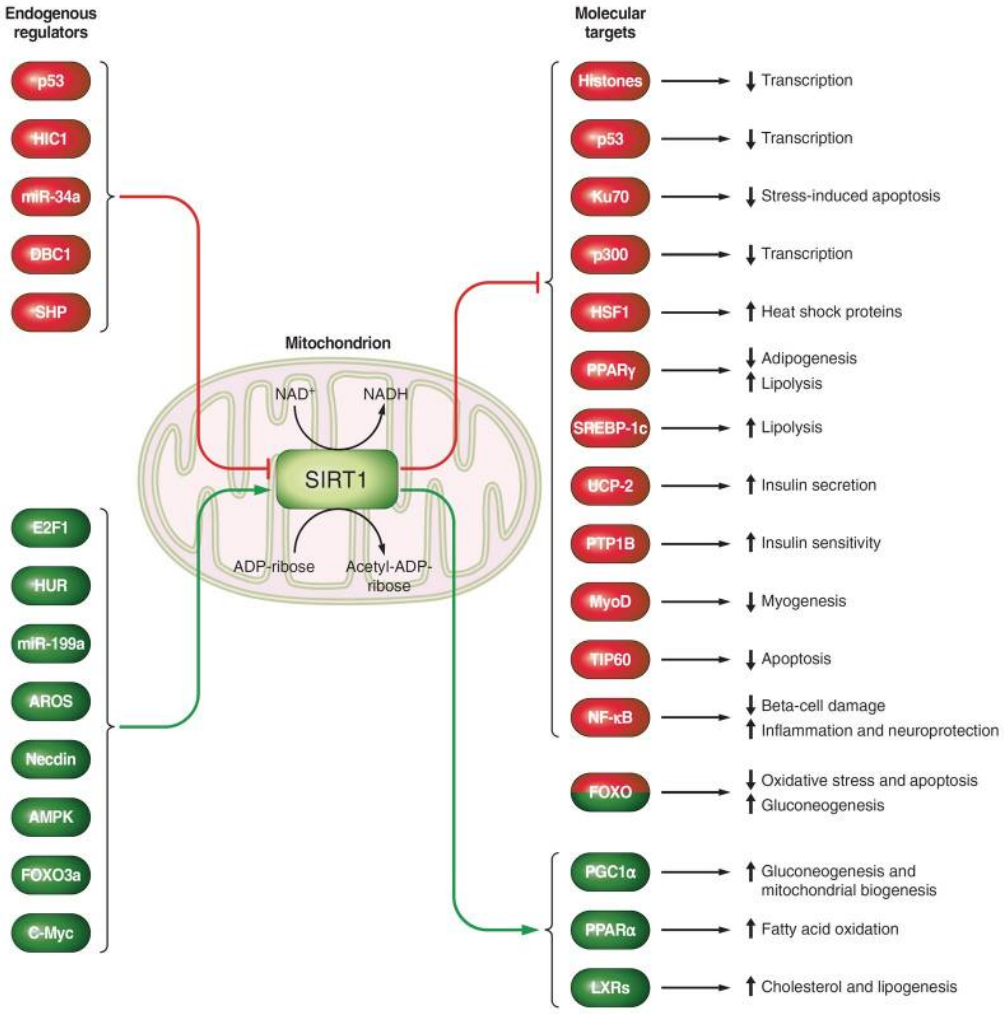

Figure 10. Overview of endogenous regulators and molecular targets of SIRT1. Taken from [149].

During energy limitations, SIRT1 levels are modulated tissue dependently. In mouse pancreatic islets, SIRT1 is suggested to exhibit decreased activity along with upregulation of Ucp2 leading to reduced ATP production and insulin secretion upon starvation [150]. In other tissues including brain, heart, muscle, white adipose tissue (WAT) and kidney, SIRT1 expression and promotor activity is upregulated during starving conditions in a Foxo3a and p53 dependent way [151], [152].

In the liver, data about SIRT1 expression and activity are somehow unclear [136]. During late stage of fasting, SIRT1-mediated activation of the transcription factor Foxo1, inhibition of STAT3 and deacetylation of the transcriptional coactivator PGC-1α induces gluconeogenesis and lowers glycolytic gene levels [153], [154], [155], [156].

Many endogenous activators and inhibitors of SIRT1 are known (Fig. 10) such as active regulator of SIRT1 (AROS), AMP-activated protein kinase (AMPK) or deleted in breast cancer 1 (DBC1). The most investigated naturally occurring activator is the polyphenol and antioxidant resveratrol (RSV) whose SIRT1 specificity is still matter of debate. RSV has been linked to improvements of mitochondrial function by activation of AMPK and PGC-1α in a SIRT1 dependent manner *in vitro* and *in vivo* [157], [158] and to the prevention and improvement of T1D or T2D in mice [159], [160], [161]. However, oral RSV administration in a recent clinical study did not affect metabolic conditions in obese patients [162].

Small molecule activators of SIRT1 were developed several years ago and were used in numerous studies showing specific activation of SIRT1 and improvements in mitochondrial and metabolic functions in animal models of T2D and in *in vitro* settings [163], [164], [160], [165]. However, safety, specificity and function of those chemical activators have been widely debated since the activator SRT1720 caused unexpected death cases and failed to have anti-diabetic effects in mice in other studies [166].

1.5.3 Role of Sirtuin 1 in diabetes

Various studies using cell and transgenic mouse models have described SIRT1 as an important beneficial metabolic regulator and key player in glucose sensing but the SIRT1 literature is full of contradictory findings.

Via inhibition of the uncoupler Ucp2, which is suppressing ATP generation in β-cells, insulin secretion is enhanced in mice overexpressing SIRT1 exclusively in β-cells, or in adipose tissue, brain and mouse embryonic fibroblasts [167], [168]. Glucose tolerance and overall metabolic conditions are improved in those mice. SIRT1 overexpression was reported to enhance energy efficiency and to prevent diabetes in mice [169]. Furthermore, specific deletion of SIRT1 in β-cells of mice leads to impaired insulin secretion and defects in mitochondrial function [170]. However, the SIRT1 target protein PGC-1α, which is suggested to increase mitochondrial biogenesis, is elevated in islets of diabetic animal models and overexpression of PGC-1α in islets can lead to β-cell dysfunction [171].

SIRT1 was shown to be a central regulator in adaptation to diet-induced metabolic dysfunctions [172], [173]. Transgenic mice lacking SIRT1 deacetylase activity are not able to cope with high-fat diet, accumulate hepatic lipids and are insulin resistant [174]. It has been shown that activation of SIRT1 by NR, a precursor of NAD^+, can protect HFD-induced metabolic damages through increased oxidative metabolism and mitochondrial function [175]. Interestingly, SIRT1 null mice, which exhibit developmental defects and sterility, have lower glucose and insulin levels and improved glucose tolerance [176], [150], [177].

Moreover, SIRT1 activation was reported to modulate insulin sensitivity in multiple and sometimes unclear ways. In the skeletal muscle, it has been suggested that SIRT1 deacetylates PGC-1α leading to peroxisome proliferator activated receptor α (PPARα) activation, increased fatty acid oxidation and improved insulin sensitization [178], [179]. However, overexpression of SIRT1 in rodent muscle was reported to downregulate PGC-1α protein expression and thereby inhibits mitochondrial biogenesis [180], despite the characterized beneficial role of SIRT1 in mitochondrial regulation [181], [154], [153]. Furthermore, HFD-induced impairment of skeletal muscle insulin sensitivity could not be

prevented by SIRT1 overexpression [182]. It was demonstrated in obese mice that the positive effects of PGC-1α overexpression on insulin sensitivity only occur in combination with exercise [183].

In the liver, overexpression of SIRT1 reduced insulin resistance in a study of Li et al. [184], whereas knockdown of hepatic SIRT1 in the context of T2D was reported to be beneficial as indicated by decreased liver glucose production and increased whole body insulin responsiveness in a rat model [185]. Furthermore, hepatic insulin sensitivity is improved in PGC1α deficient mice [186].

In WAT, SIRT1 inhibits PPARγ and thereby suppresses adipogenesis and fat storage and promotes lipolysis in response to nutrient restriction [187]. Most recently, it was shown that knock-out of SIRT1 in adipocytes promotes PPARγ activity and thereby increases glucose tolerance and insulin sensitivity during chronic HFD [188]. Brown adipocyte function can be potentiated by SIRT1 leading to improved glucose tolerance and insulin sensitivity [189].

Oxidative stress is an essential factor in the pathogenesis of diabetes. Prolonged exposure induces chronic inflammation by activation of transcription factors such as NF-κB, AP-1 or HIF-1α leading to the expression of various pro-inflammatory genes [190], [191]. Pancreatic β-cells are protected from oxidative stress and the induction of pro-inflammatory cytokines via SIRT1-mediated deacetylation of Foxo [192], [193] and NF-κB as demonstrated in isolated rat islets [194]. SIRT1 is able to induce the production of antioxidant enzymes leading to the inhibition of HFD-induced NF-κB activation [172]. Also in other tissues such as kidney cells, adipocytes or macrophages, SIRT1 protects from inflammatory processes and improves insulin signaling [195], [196], [197]. Nevertheless, overexpression of SIRT1 was shown to enhance TNFα-induced apoptosis [198] and SIRT1 inhibition improved hyperglycemia in diabetic rats, despite elevation of inflammation [199]. Altogether, apparently contradictory effects reported in the literature point to tissue- and context-specific effects of SIRT1 precluding a generalization of SIRT1 functions.

Additional findings support the ambivalent role of SIRT1 in the development of diabetes. Histone deacetylase (HDAC) inhibitors are emerging as new modulators of glucose and fatty acid metabolism in the treatment of diabetes. HDAC1 and 2 inhibitors have beneficial effects on insulin sensitivity and β-cell function, partly via enhancement of AMPK activity [200]. HDAC3 inhibitors were reported to increase PPARγ function with effects similar to thiazolidinediones used in diabetes treatment, and to inhibit inflammation in obese mice [201], [202]; however, also negative metabolic effects were described [203]. Thus, inhibiting the function of SIRT1 as a histone deacetylase may also be beneficial in metabolic diseases.

A growing body of evidence points to a central role of SIRT1 in the development of T1D. In the mldSTZ model, a T1D model in animals, it is proposed that NAD^+ is depleted by activation of PARP and DNA repair, leading to β-cell death. In line with that, resistance to STZ-induced diabetes has been shown in PARP-1 knock-out mice [204], [205]. Several studies have proven that nicotinamide, a precursor of NAD^+ and at the same time end-product inhibitor of SIRT1, protects against STZ-induced β-cell damage and diabetes in rodents, T1D patients and in individuals with high risk [206], [207], [208], [209], [210] but it failed to prevent T1D in a large clinical trial [211]. It is still matter of debate if the inhibition of SIRT1 or PARP inhibition along with re-filling of the NAD^+ stocks is predominant in causing the nicotinamide-driven effects [212], [213]. However, a nicotinamide derivative, isonicotinamide, which antagonizes nicotinamide-induced SIRT1 inhibition protects against STZ-induced hyperglycemia and β-cell damage [214]. Furthermore, SIRT1 null mice are more prone to mldSTZ-induced autoimmune diabetes [215], whereas mice overexpressing SIRT1 in the β-cells have increased susceptibility to STZ compared to heterozygous SIRT1 knock-out mice [216].

1.5.4 Sirtuin 1 and the immune system

SIRT1 has mainly been linked to metabolism but it is increasingly recognized as regulator of immune responses and inflammation. SIRT1 is highly expressed in dendritic cells, activated and anergic T-cells and various other immune cells [217], [218]. Several studies have focused on the role of SIRT1 in inflammation and the activation and differentiation of T-cells but the mechanisms are still poorly understood.

Knock-out animal models lacking SIRT1 in myeloid cells, $CD4^+$ T-cells or dendritic cells revealed the central role of SIRT1 in the modulation of both the innate and the adaptive immune system. Myeloid-specific knock-out of SIRT1 led to hyperacetylation of the NF-kB subunit p65 and thereby caused the induction of proinflammatory genes and insulin resistance in animals fed a HFD [219]. Consistent with these data, activation of SIRT1 was shown to inhibit inflammation via deacetylation of the transcription factors AP-1 and NF-kB at lysine residues [220], [198]. Further supporting, SIRT1 knock-out in peritoneal macrophages *in vitro* increased LPS-stimulated inflammation and SIRT1 activation in Zucker fatty rats improved glucose tolerance and insulin sensitivity by decreasing tissue inflammation [196]. Deletion of SIRT1 in dendritic cells exhibited a regulatory role of SIRT1 in Th1 and Treg differentiation through the modulation of IL-12 and TGF-β expression, respectively [217]. However, Sirt1-null dendritic cells have enhanced ability to produce IL-27 and IFN-β leading to the inhibition of Th17 differentiation [221]. Interestingly, SIRT1 contributes to immunometabolic reprogramming during acute inflammation by increasing fatty acid oxidation and thereby supporting adaptation responses [222].

SIRT1 is able to inhibit transcription and production of IL-2, which is a main factor for the development of functional effector T-cells and which is able to reverse T-cell anergy and tolerance [223], [218], [224]. In parallel, IL-2 is a central regulator for the suppressive function of Tregs and therefore immune tolerance [225], [21]. In former studies, cyclosporine was used as immunotherapeutical treatment against IL-2-mediated autoreactive T-cell activation in T1D patients but the beneficial effects were only temporary [226]. Interestingly, the lack of IL-2 has been shown to play a crucial role in the development of T1D [227] and therapies with low-dose IL-2 treatment to boost or restore Tregs protected

from the development of T1D in NOD mice and in clinical studies [228], [229]. β-cell specific IL-2 therapy increased the islet Tregs pool and thereby prevented T1D in NOD mice [230]. In other autoimmune conditions, such as Hepatitis C virus-induced vasculitis, low-dose IL-2 treatment in a clinical trial led to Treg recovery and clinical improvements [231].

The transcription factor Foxp3, which is crucial for the development and function of Tregs, was deacetylated by SIRT1 leading to proteasomal degradation [23], whereas blockage of SIRT1 improved Treg suppressive function *in vitro* and *in vivo* [232]. It has been convincingly shown in a recent study that SIRT1 deacetylates the transcription factor retinoic acid receptor-related orphan receptor-γ t (RORγt), which is regulating the differentiation of Th17 effector cells, and thereby enhances the Th17/Treg ratio and the susceptibility to autoimmune diseases [233].

Additionally, inhibition of HDACs in autoimmune diseases including colitis was suggested to improve Treg function [234], [235]. There is evidence that also the deacetylase SIRT1 influences the development of colitis. Intestinal deletion of SIRT1 protected from colitis and colitis-induced colorectal cancer by re-arranging the gut microbiome [236] and pharmacological inhibition of SIRT1 increased Treg formation and attenuated colitis [237].
In contrast, whole body Sirt1 -/- mice develop an autoimmune-like phenotype with increased anti-nuclear antigen antibodies [238]; however, they are developmentally affected, infertile and have a small body size. Most recently, SIRT1 was also associated with the regulation of autoimmune regulator (AIRE)-mediated immunological tolerance in the thymus [239].
Despite the conflicting data, all of this is indicative for an essential role of SIRT1 in T-cell biology and the context of autoimmune diseases including T1D and colitis.

1.5.5 Sirtuin 1 mutation L107P in familial type 1 diabetes

Recently, a SIRT1 point mutation (L107P) was described in a family of Ashkenazy Jews as a first monogenic form of diabetes by our group, among others [215]. As shown in Figure 11, the thymidine to cytosine point mutation in exon 1 of the *Sirt1* gene leads to an exchange of the amino acid leucine to proline at residue 107 (L107P). Five members of this family carrying one mutated allele have autoimmune diseases; four have type 1 diabetes and one has ulcerative colitis. The mutation is located outside of the catalytic core but in the amino terminal region, which is involved in protein-protein interactions. However, the top known SIRT1 substrates were not influenced in interaction assays. Cultured myotubes of the index patient displayed reduced glucose uptake in response to insulin and the number of regulatory T-cells was decreased in the blood of the patient, compared to healthy controls and other type 1 diabetes patients. Based on these findings, we generated INS-1E cells ectopically overexpressing the mutated human SIRT1 and knock-in mice carrying the corresponding mouse mutation to further understand the role of SIRT1 in the development of autoimmune diseases including T1D and colitis.

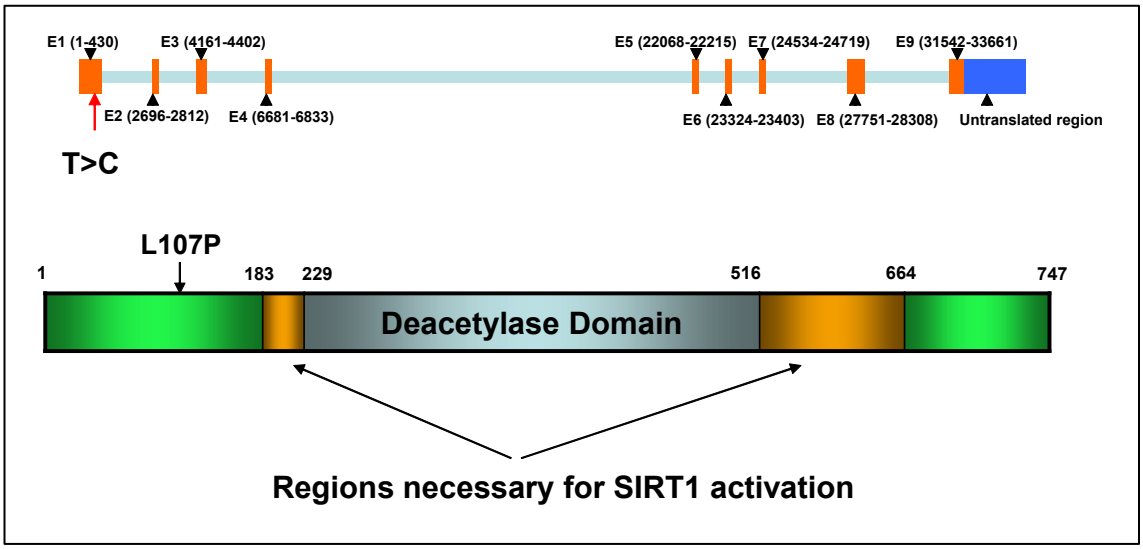

Figure 11. Characterization of the SIRT1 mutation L107P associated with diabetes and colitis. *Sirt1* gene structure with thymidine to cytosine point mutation (red arrow) and predicted protein structure with the amino acid exchange leucine to proline at residue 107 (L107P black arrow). Taken from [215].

2 Aim of the study

In this study, we investigated the modulatory effects of angiotensin II (Ang II) and sirtuin 1 (SIRT1) on inflammation and the immune system in the development of diabetes. The thesis is divided in three parts.

The first published part focuses on the effects of Ang II on islet inflammation and β-cell dysfunction in the context of T2D. Much research was done on the role of the renin-angiotensin system (RAS) in the context of hypertension. Further, it was shown in clinical studies and animal models that RAS inhibition has protective effects in the context of T2D. However, little is known about the direct effects of Ang II on pancreatic islets and the responsible mechanisms in the pathogenesis of diabetes. We specifically wanted to examine inflammatory pathways underlying the effects of Ang II in the development of T2D, independently of vasoconstriction.

Second and third part of this work describe the immunomodulatory functions of SIRT1 in the context of diabetes, primarily autoimmune T1D. Commonly, SIRT1 is implicated with regulations of metabolism and longevity but the involvement in autoimmunity and inflammation is less investigated. A family carrying a point mutation in the *Sirt1* gene (L107P) was associated with autoimmune diseases, including T1D and ulcerative colitis. We wanted to translate the phenotype of the patients to an INS-1E cell model and a knock-in mouse model carrying the corresponding SIRT1 mouse mutation (L102P) to further understand the underlying mechanisms of this mutation and the general link between SIRT1, glucose metabolism and immune responses. Finally, we used inducible β-cell specific SIRT1 knock-out mice to test the impact of SIRT1 on β-cell metabolism and diabetes, without affecting the development of the mice. Based on the literature, little is known about the connection of SIRT1 modulation and the development of T1D. The aim of this part was to characterize the distinct role of β-cell SIRT1 in inflammation and the initiation and progression of T1D.

3 Angiotensin II induces inflammation in the context of type 2 diabetes

3.1 Publication: "Angiotensin II Induces Interleukin-1β-Mediated Islet Inflammation and β-Cell Dysfunction Independently of Vasoconstrictive Effects"

In this publication Nadine S. Sauter (N.S.S.) and Constanze Thienel (C.T.) share the first authorship. Majority of planning and performing experiments was done by N.S.S. The contribution of C.T. to this work includes: performing glucose-stimulated insulin secretion experiments, oxygen consumption assays, determination of immune cell infiltration in islets, helping with *in vivo* and *in vitro* experiments like mouse islet assays, as well as writing assistance and revision of the manuscript.

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Angiotensin II Induces Interleukin-1β–Mediated Islet Inflammation and β-Cell Dysfunction Independently of Vasoconstrictive Effects

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Pathological activation of the renin-angiotensin system (RAS) is associated with the metabolic syndrome, and the new onset of type 2 diabetes can be delayed by RAS inhibition. In animal models of type 2 diabetes, inhibition of the RAS improves insulin secretion. However, the direct effects of angiotensin II on islet function and underlying mechanisms independent of changes in blood pressure remain unclear. Here we show that exposure of human and mouse islets to angiotensin II induces interleukin (IL)-1–dependent expression of IL-6 and MCP-1, enhances β-cell apoptosis, and impairs mitochondrial function and insulin secretion. In vivo, mice fed a high-fat diet and treated with angiotensin II and the vasodilator hydralazine to prevent hypertension showed defective glucose-stimulated insulin secretion and deteriorated glucose tolerance. Application of an anti–IL-1β antibody reduced the deleterious effects of angiotensin II on islet inflammation, restored insulin secretion, and improved glycemia. We conclude that angiotensin II leads to islet dysfunction via induction of inflammation and independent of vasoconstriction. Our findings reveal a novel role for the RAS and an additional rationale for the treatment of type 2 diabetic patients with an IL-1β antagonist.

Obesity and type 2 diabetes are related to hypertension and to increased activation of the renin-angiotensin system (RAS) (1–3). Multiple trials have shown that RAS blockade reduces the incidence of new-onset type 2 diabetes in high-risk populations (4). On the basis of several meta-analyses, this reduction ranges between 22% and 30% (5,6). In addition, in different animals models of type 2 diabetes, treatment with either angiotensin-receptor blockers or ACE inhibitors improves glucose tolerance and β-cell function (2,7–10). All of this suggests a role for angiotensin II in the development of type 2 diabetes.

The RAS is classically known as a systemic hormonal system regulating blood pressure, fluid balance, and electrolyte absorption (11). Finding a local RAS in various tissues and organs such as brain, kidney (12), heart (13), liver, and adipose tissue (14) has expanded its role to diverse physiological functions in addition to its effects on circulation. All key components of the RAS also have been localized to the endocrine pancreas, including the precursor angiotensinogen and the angiotensin II type 1 receptor (15,16). Furthermore, obesity and hyperglycemia increases the expression of the local RAS in pancreatic islets (17), adipose tissue (18), and skeletal muscle.

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See accompanying article, p. 1094.

Several hypotheses of how RAS activation might contribute to the development of diabetes and why its blockade could be protective have been suggested. In diabetic animal models, angiotensin II leads to decreased blood flow in insulin target tissues and pancreatic islets, which results in reduced insulin and glucose delivery (19–21). In skeletal muscle angiotensin II interferes with glucose uptake by decreasing GLUT4 translocation to the plasma membrane (22) and induces insulin resistance (23). RAS inhibition prevents these effects in vivo, resulting in increased glucose tolerance and improved islet function (2,8,9,24). However, whether the amelioration of metabolic parameters is a consequence of normalization of vasoconstriction or due to inhibition of local RAS is unclear. Studies of isolated islets treated with angiotensin II or its blockers point to a possible role of activated local RAS or direct angiotensin II effects on impaired insulin secretion (15,17,25).

It has been recognized more recently that in type 2 diabetes chronic inflammation is involved in the dysfunction of pancreatic islets (26,27). Increased numbers of immune cells were observed in pancreatic islets of animals fed a high-fat diet (HFD) and of patients with type 2 diabetes (28). There is increasing evidence that islet inflammation is mediated by an imbalance of interleukin (IL)-1β and its naturally occurring antagonist IL-1 receptor antagonist (IL-1Ra). This contributes to the formation of insulitis by recruiting and activating IL-1β–producing macrophages. Treatment of type 2 diabetes or obese patients with anakinra, the recombinant form of IL-1Ra, or specific anti–IL-1β antibodies improved glycemia and β-cell function and reduced circulating inflammatory indicators (29–34).

Some observations point to a possible proinflammatory role for angiotensin II (35,36). It triggers inflammatory processes in the kidney (37) and induces the chemokine MCP-1 in pancreatic cancer cells (38). In blood mononuclear cells, ACE inhibitors suppress IL-1 and tumor necrosis factor (TNF) synthesis (39). Furthermore, in clinical trials treatment with angiotensin II receptor antagonists reduced the proinflammatory markers TNF-α, IL-6, and CRP (40,41), as well as MCP-1, in the circulation of patients with cardiovascular diseases (42). Similarly, in HFD-fed mice, decreased serum concentrations of interferon-γ and MCP-1 and diminished proinflammatory gene expression in pancreatic islets were observed with angiotensin II receptor antagonists or ACE inhibitors (24,43).

All of this suggests a role for angiotensin II in the development of inflammation and type 2 diabetes. However, a possible direct effect of angiotensin II on metabolism and insulin secretion independent of changes in blood pressure and the underlying pathway remain to be investigated.

RESEARCH DESIGN AND METHODS

Human Pancreatic Islets

Human islets were isolated from pancreata of cadaver organ donors in the islet transplantation centers in Lille (France) and Geneva (Switzerland), in accordance with the local institutional ethical committee. Human islets were provided by the islets for research distribution program through the European Consortium for Islet Transplantation, under the supervision of the Juvenile Diabetes Research Foundation (31-2012-783). Islets were cultured in CMRL-1066 medium containing 5 mmol/L glucose, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamax, and 10% FCS (Invitrogen, Basel, Switzerland) on extracellular matrix–coated 24-well plates (Novamed Ltd, Jerusalem, Israel) in a humid environment containing 5% CO_2. Islets were treated for 96 h (media were renewed after 48 h) with or without 1 μmol/L angiotensin II (A9525; Sigma Aldrich, Switzerland), 1 μg/mL IL-1Ra (Kineret; Amgen, Thousand Oaks, CA), and/or 10 μmol/L IKK-2 inhibitor (SC-514; Merck, Darmstadt, Germany). Culture supernatants were collected and islets were used for RNA extraction or glucose-stimulated insulin secretion experiments.

Mouse Pancreatic Islets

To isolate mouse islets, pancreata were perfused with a collagenase solution (Worthington, Lakewood, NJ) and digested in the same solution at 37°C, followed by filtration through 500- and 70-μm cell strainers. Islets were cultured in RPMI-1640 medium containing 11.1 mmol/L glucose, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamax, 50 μg/mL gentamicin, 1:1,000 Fungizone (Gibco), and 10% FCS. Islets were directly collected for RNA extractions, were cultured for 36 h on extracellular matrix–coated 24-well plates and treated for 24 h with 1 μmol/L angiotensin II before RNA extraction and protein measurements in the supernatant, or were used for glucose-stimulated insulin secretion experiments (48 h treatment with 1 μmol/L angiotensin II).

INS-1E Cell Culture

INS-1E cells (44) were cultured in RPMI-1640 medium containing 11.1 mmol/L glucose, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamax, 10% FCS, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, and 50 μmol/L 2-mercaptoethanol. For experiments, 100,000 cells/well were seeded in 24-well plates. Cells were treated with or without 1 μmol/L angiotensin II and/or 10 μmol/L IKK-2 inhibitor for 72 h before RNA extraction. For glucose-stimulated insulin secretion experiments, cells were treated 96 h with 1 μmol/L angiotensin II. Apoptosis rates were measured with the Cell Death Detection Elisa Kit (Roche Diagnostics, Switzerland) according to the manufacturers' instructions.

Glucose-Stimulated Insulin Secretion Assay

For in vitro or ex vivo glucose-stimulated insulin secretion experiments, islets or INS-1E cells were seeded for 2 days in quadruplicate. After treatment with angiotensin II, supernatants were collected and stored at −20°C (chronic insulin release). Cells were preincubated for 30 min in modified Krebs-Ringer bicarbonate buffer (mKRBB; 115 mmol/L sodium chloride, 4.7 mmol/L potassium chloride,

2.6 mmol/L calcium chloride dihydrate, 1.2 mmol/L monopotassium sulfate, 1.2 mmol/L magnesium sulfate heptahydrate, 10 mmol/L HEPES, and 0.5% bovine serum albumin [pH 7.4]) containing 2.8 mmol/L glucose. mKRBB was then replaced by mKRBB 2.8 mmol/L glucose for 1 h (basal insulin release), followed by 1 h in mKRBB 16.7 mmol/L glucose (stimulated insulin release). Islets or INS-1E cells were extracted with 0.18 N hydrogen chloride in 70% ethanol to determine insulin content. Insulin concentrations were determined using human or mouse insulin ultrasensitive ELISA (Mercodia, Uppsala, Sweden) or mouse/rat insulin kit (Mesoscale Discovery, Rockville, MD). Stimulatory index was determined as ratio of insulin secretion at 16.7 to that at 2.8 mmol/L glucose/hour.

Oxygen Consumption Assay

Oxygen consumption rates were determined using the Seahorse extracellular flux analyzer XF^{e}96 (Seahorse Bioscience). INS-1E cells (145,000 cells/well) were seeded on poly-D-lysine-treated Seahorse 96-well microplates in 175 μL INS-1E medium/well 2 days before the experiment. Medium was changed and cells were treated with 1 μmol/L angiotensin 24 h before the experiment. At the day of the assay, cells were preincubated in unbuffered assay medium (RPMI-1640 [R6504; Sigma] supplemented with 11.1 mmol/L glucose) for 1.5 h at 37°C in air. To test ATP turnover, maximal respiratory capacity, and nonmitochondrial respiration of the cells, 1 μmol/L oligomycin, 2 μmol/L of the uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone, and 1 μmol/L rotenone were successively injected.

TUNEL Assay

To determine β-cell apoptosis, human islets were dispersed with trypsin-EDTA (Invitrogen) for 6 min at 37°C. Single cells were cultured and treated with angiotensin II for 4 days (new medium and treatment were added after 2 days). Subsequently, the cells were fixed in 4% paraformaldehyde for 30 min, followed by incubation in 0.5% Triton-X-100 for 4 min. Cells were triple stained with the terminal deoxynucleotidyl TUNEL technique (In Situ Cell Death Detection Kit; Roche Diagnostics, Switzerland), a polyclonal antibody against insulin (Dako, Denmark), and nuclear DAPI (Sigma).

RNA Extraction and Quantitative PCR

Total RNA of isolated human or mouse islets and INS-1E cells was extracted using the Nucleo Spin RNA II Kit (Machery Nagel, Düren, Germany). cDNA was prepared with random hexamers and Superscript II (Invitrogen). For quantitative PCR, the real-time PCR system 7500 (Applied Biosystems) and the following TaqMan assays were used: human IL-1β, Hs00174097_m1; IL-6, Hs00174131_m1; IL-8, Hs00174103_m1; 18s, Hs99999901_s1; MCP-1, Hs00234140_m1; rat CXCL1, Rn00578225_m1; and mouse GAPDH, Mm99999915_g1; IL-6: Mm00446190_m1. Gene expression was analyzed using the comparative $2^{-\Delta\Delta CT}$ method.

Cytokine Assays

Human or mouse IL-6 concentrations in islet culture supernatants or plasma were assayed using Luminex technology (Millipore, Billerica, MA) or Mesoscale kits (Mesoscale Discovery).

Immunohistochemical Stainings

Formalin-fixed pancreata were embedded in paraffin and blocks were cut at 50-μm intervals. Tissue sections were deparaffinized, rehydrated, and stained with rat antimouse CD45 (BD Bioscience, New Jersey), rat antimouse F4/80 (Cedarlane, Burlington, ON), or rabbit antimouse CD3 antibody (Abcam, Cambridge, U.K.). To determine immune cell infiltration, two to five slides per mouse were stained; CD45$^+$ cells were counted under a microscope (Olympus BX63) and the percentages of affected islets per mouse were calculated. In the angiotensin II and hydralazine group, 862 islets were counted and 28 islets were affected (10 mice, pooled from three experiments); in the angiotensin II and hydralazine plus anti–IL-1β antibody group, 458 islets were counted (four mice; eight islets were affected).

Animal Experiments

Male C57BL/6N mice were obtained from Charles River Laboratories (Sulzfeld, Germany) at 4 weeks of age. For the first three experiments, a total of 78 mice were used; in the fourth experiment 17 mice were used. They were fed an HFD (D12331; Research Diets, New Brunswick, NJ), and after 12 weeks they were subcutaneously implanted with osmotic mini pumps (Alzet 2004; Durect Corp., Cupertino, CA) releasing either angiotensin II (1 μg/kg/min; A9525; Sigma) or saline for 4 weeks. Thirty-nine animals in the first three experiments and all 17 animals in the fourth experiment received hydralazine (H1753; Sigma) in their drinking water at a concentration of 250 mg/L. Animals used in the fourth experiment were injected subcutaneously once per week for 4 weeks with either saline (five mice), control antibodies (anti-cyclosporine-A; four mice), or mouse anti–IL-1β antibodies at a concentration of 10 μg/g (eight mice). The anti–IL-1β antibody is a mouse antibody with the same specificity as canakinumab (45). Antibodies were kindly provided by Novartis (Basel, Switzerland). Surgery was done in a specific pathogen–free environment, animals were anesthetized with Ketalar (65 mg/kg) and Xylasol (13 mg/kg) by intraperitoneal injection. To prepare for pump implantation, an air pocket was created under the skin. The sterile pumps (filled with saline or angiotensin II) were inserted in the pocket and the wound was closed with two clips. After the first signs of waking, an analgesic (Temgesic, 0.05 mg/kg) was injected subcutaneously. After 24 h and, if necessary, 48 h, another injection of painkiller was given. Wound healing and the health state of every mouse was observed and recorded on a score sheet every day for 1 week. All animals were housed singly in cages in a temperature-controlled room with a 12-h light/12-h dark cycle and were allowed free access to

food and water, according to Swiss veterinary law and institutional guidelines. After 4 weeks of treatment mice were used for glucose/insulin tolerance tests. After death, heart blood was collected and islets were isolated for RNA expression or ex vivo glucose-stimulated insulin secretion experiments, or pancreata were taken for histology.

Blood Pressure Measurements

Blood pressure was measured with a tail-cuff system (Visitech Systems, Apex, NC) in six to eight mice per group. Mice were first habituated to the tail-cuff system for 5 days to avoid stress artifacts caused by the demanding procedure. Every day they were put into the system and the tails were fixed in a cuff. After training the mice, data were acquired on the following 3–5 days. While discarding the first 10 measurements, the subsequent 10 measurements were averaged and reported.

Glucose and Insulin Tolerance Tests

For intraperitoneal glucose tolerance tests, mice were fasted for 6 h in the morning and injected intraperitoneally with 2 g glucose/kg body weight. Blood samples for glucose measurements were obtained at 0, 15, 30, 60, 90, and 120 min using a glucometer (Freestyle; Abbott Diabetes Care Inc., Alameda, CA) and at 0, 15, and 30 min for measurement of plasma insulin concentrations using an insulin ELISA (Mercodia). For intraperitoneal insulin tolerance tests, mice were fasted 3 h in the morning before administration of 1 unit/kg insulin (Novo Nordisk, Bagsvaerd, Denmark), and blood glucose was measured at 0, 15, 30, 60, and 90 min.

Statistics

Statistical analysis was performed using GraphPad Prism 5 (Graphpad Software Inc., San Diego, CA). Data are presented as mean ± SEM and were analyzed using one- or two-way ANOVA or unpaired Student *t* test. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Angiotensin II Has Deleterious Effects on Human and Mouse Islet β-Cells

To investigate the effect of angiotensin II on β-cell function and survival in vitro, we exposed isolated human and mouse islets to 1 μmol/L angiotensin II for 48 to 96 h. This treatment resulted in a slight increase of basal and a decrease of glucose-stimulated insulin secretion (Fig. 1*A* and *C*), leading to a significant decrease of the stimulatory index overall compared with control islets (Fig. 1*B* and *D*). To discriminate whether the observed effects in whole islets are the result of (partly) direct signaling of angiotensin II on β-cells or indirect via paracrine actions, we used the β-cell line INS-1E and observed significantly higher basal insulin secretion and reduction of the stimulatory index following exposure to angiotensin II (Fig. 1*E* and *F*), as well as diminished mitochondrial respiratory capacity (Fig. 1*G*). Staining of treated and untreated single human islet cells with the TUNEL assay revealed a threefold increase of angiotensin II–induced β-cell apoptosis (Fig. 1*H* and *I*). Similarly, exposure of INS-1E cells to 1 μmol/L angiotensin II for 3 days induced a 1.3-fold increase of apoptosis (data not shown).

Angiotensin II Induces Cytokine Expression and Release in Human and Mouse Islets

Next we questioned whether angiotensin II has proinflammatory effects in isolated human and mouse islets. Angiotensin II increased the gene expression of several cytokines, including IL-1β, MCP-1, IL-8, and IL-6 (Fig. 2*A*–*D*). This led to elevated IL-6 protein release into the culture supernatant of human islets (Fig. 2*E*). Similarly, IL-6 expression and release was stimulated by angiotensin II in mouse islets (Fig. 2*F* and *G*). TNF was not significantly changed by angiotensin II, whereas interferon-γ was not detectable (not shown). In the β-cell line INS-1E induction of the chemokine CXCL1 (functional homolog to human IL-8 [CXCL8]) (Fig. 2*H*) could be detected. Angiotensin II–induced increase in IL-1β and IL-6 in human islets and increased *CXCL1* gene expression in INS-1E cells was mediated by nuclear factor (NF)-κB; it was fully prevented by the addition of an IκB-kinase-2 inhibitor (Fig. 3*A*–*C*). Finally, blocking IL-1 signaling with IL-1Ra prevented the angiotensin II–induced increase in *IL-6* gene expression (Fig. 3*D*) and release (Fig. 3*E*), demonstrating that elevated IL-6 concentrations depend on IL-1 signaling.

Angiotensin II Treatment Impairs Glucose Tolerance Independently of Its Vasoconstrictive Effect

To investigate the role of angiotensin II in the development of impaired insulin secretion in the context of type 2 diabetes, mice were fed an HFD for 12 weeks; then, osmotic pumps releasing either saline or angiotensin II for 4 weeks were implanted. Exposure to angiotensin II leads to vasoconstriction and hypertension, which may affect glucose tolerance because of altered glucose and insulin delivery to the insulin-sensitive tissues. Therefore, half of the animals also received the vasodilator hydralazine in the drinking water (Fig. 4*A*). As shown in Fig. 4*B*, vasoconstrictive effects of angiotensin II were present and hydralazine application reduced the angiotensin II–induced increase in mean arterial pressure. Mean body weight did not change in mice receiving angiotensin II or hydralazine alone compared with the saline group and was reduced in mice treated with the combination of the two compared with both the saline and the angiotensin II groups alone (Fig. 4*C*).

To assess glucose metabolism, we performed intraperitoneal glucose tolerance tests after 16 weeks on an HFD and angiotensin II treatment during the last 4 weeks. In mice with angiotensin II treatment alone, blood glucose as well as plasma insulin concentrations increased only marginally after intraperitoneal administration of glucose because vasoconstriction leads to reduced glucose absorbance. This is supported by an intravenous glucose tolerance experiment where glucose tolerance was reduced in angiotensin II–infused animals (data not shown). As

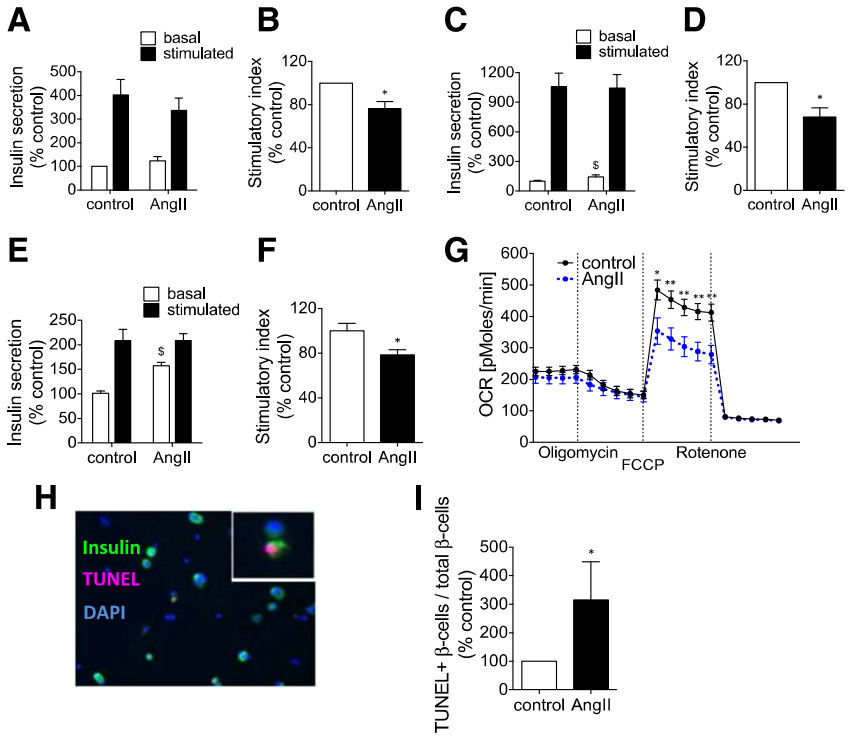

Figure 1—Angiotensin II (AngII) inhibits insulin secretion in human and mouse islets and induces β-cell apoptosis. Glucose-stimulated insulin secretion and corresponding stimulatory index (ratio of stimulated to basal glucose-stimulated insulin secretion) in human (A and B) and mouse islets (C and D) and INS-1E cells (E and F), as well as oxygen consumption rates of INS-1E cells (G) after exposure to 1 μmol/L AngII for 1 day (INS-1E, G), 2 days (mouse islets), or 4 days (human islets and INS-1E; E and F). FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone. (H) Representative image of TUNEL, insulin, and DAPI triple staining of dispersed human islet cells. (I) TUNEL-positive β-cells following the same culture condition. Statistics were performed using the Student t test. Bar graphs show data as mean ± SEM. Data are averages of three to six independent experiments. $P < 0.05 compared with basal control, *P < 0.05, **P < 0.01.

shown in Fig. 4*D* and *E*, compared with saline, hydralazine improved glucose tolerance and insulin secretion in mice fed an HFD. In contrast, treatment with angiotensin II plus hydralazine led to highly impaired glucose tolerance along with a complete lack of glucose-stimulated insulin secretion (Fig. 4*D* and *E*). To assess whether increased insulin resistance contributed to impaired glucose metabolism in the combined angiotensin II and hydralazine group, we performed insulin tolerance tests (Fig. 4*F*). Even in the presence of angiotensin II, hydralazine improved insulin sensitivity compared with the saline group (Fig. 4*F*). Therefore, the glucose intolerance observed with angiotensin II and hydralazine treatment is a result of β-cell failure and is not caused by impaired insulin sensitivity.

Angiotensin II Infusion Elevates Circulating and Islet-Derived IL-6

After 16 weeks of HFD feeding and 4 weeks of treatment with angiotensin II and hydralazine, IL-6 plasma concentrations were increased in treated mice compared with control animals or mice receiving hydralazine alone (Fig. 4*G*). In contrast to the in vivo situation, ex vivo glucose-stimulated insulin secretion assays revealed no differences in basal and glucose-stimulated insulin concentrations in the various groups (Fig. 4*H*). However, chronic insulin release over 36 h into the culture medium containing 11.1 mmol/L glucose was significantly elevated in islets isolated from the angiotensin II and hydralazine group (Fig. 4*I*), along with increased expression of IL-6 (Fig. 4*J*), but the latter did not reach statistical significance.

Impaired Insulin Secretion Upon Angiotensin II Infusion Is Mediated by IL-1β

Since angiotensin II induces IL-1β in vitro, leading to IL-6 expression, we next investigated whether the angiotensin II–induced impaired insulin secretion could also be mediated by IL-1β in vivo. For this, mice fed an HFD for 16 weeks were treated with angiotensin II and hydralazine for the last 4 weeks, as in the previous experiments. In addition, they were injected with either specific antibodies against IL-1β or with saline or control antibodies (anti-cyclosporine-A), as shown in Fig. 5*A*. Glucose tolerance tests revealed that inhibiting IL-1β improved glycemia and restored insulin secretion compared with control groups (glucose tolerance tests of animals treated with saline and control antibodies were identical and therefore

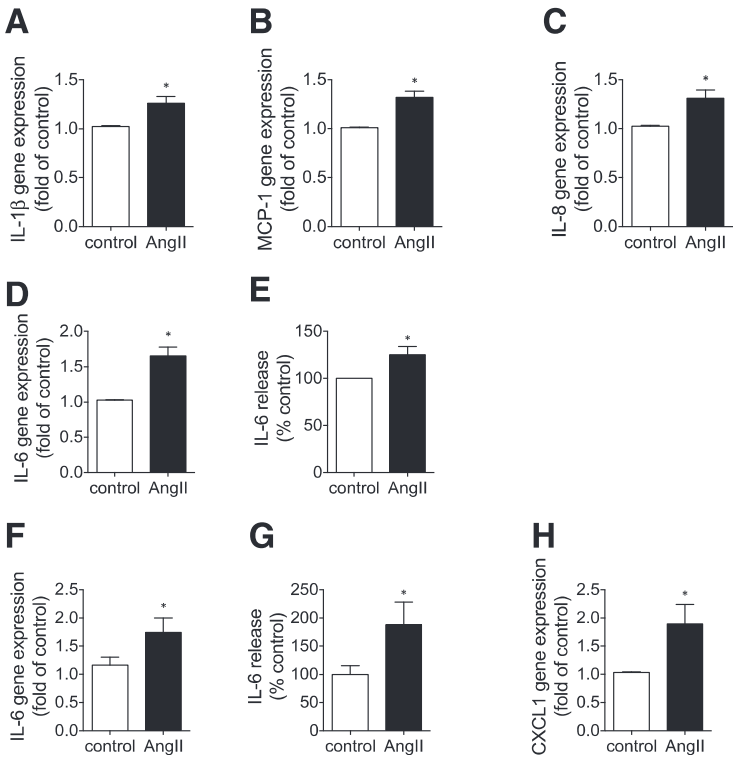

Figure 2—Angiotensin II (AngII) induces proinflammatory cytokines in human and mouse islets. Human (A–E) and mouse islets (F and G) and INS-1E cells (H) were exposed to 1 μmol/L AngII for 1 day (mouse islets) or 4 days (human islets and INS-1E) and tested for expression of IL-1b (A), MCP-1 (B), IL-8 (C), and IL-6 genes (D and F) and for IL-6 protein release (E and G), as well as for expression of the CXCL1 gene (H). Statistics were performed using the Student t test. Bar graphs show data as mean ± SEM. Human data are averages of at least 25 independent experiments, mouse data are averages of 5 experiments, and INS-1E cells are averages of at least 4 experiments. *P < 0.05.

pooled; Fig. 5*B* and *C*). The improvement in metabolism was not caused by changes in insulin sensitivity (Fig. 5*D*). Ex vivo, islets isolated from mice treated with anti–IL-1β antibodies had significantly lower basal and higher glucose-stimulated insulin secretion compared with islets from animals treated with saline or control antibodies, resulting in an improved stimulatory index (Fig. 5*E* and *F*).

Immunohistochemical stainings of pancreata with the panimmune cell marker CD45 revealed differences in immune cell infiltration (patterns of insulitis; examples are shown in Fig. 5*H–K*) in islets of mice treated with anti–IL-1β antibodies compared with the angiotensin II and hydralazine control group. The number of islets with mild forms of infiltration, as well as the total percentage of affected islets per mouse, were reduced in mice treated with angiotensin II, hydralazine, and anti–IL-1β antibodies compared with angiotensin II and hydralazine alone (Fig. 5*G*). Immunohistochemical stainings of CD3 and F4/80 indicated that infiltrating cells were mostly T cells and not macrophages (data not shown).

DISCUSSION

In this study we demonstrate that angiotensin II deteriorates glucose metabolism through deleterious effects on pancreatic β-cell mitochondrial function and insulin secretion. This effect involves IL-1β– and NF-κB–mediated inflammation and apoptosis and is independent of changes in blood pressure and insulin sensitivity.

In vivo infusion of angiotensin II for 4 weeks completely abolished glucose-stimulated insulin secretion. Surprisingly, after isolation and culturing, islets from angiotensin II–treated mice showed similar insulin secretion upon glucose stimulation as islets from saline-infused mice. Several possibilities can explain this finding: Angiotensin II–induced damage is reversible, and the islets had recovered between isolation and the insulin secretion assay. Alternatively, the in vivo deleterious effect of angiotensin II may be mediated via immune cells, which are lost following islet isolation. This would explain the discrepancy between the mild, direct in vitro effects of angiotensin II (Fig. 1) on insulin secretion compared with the strong in vivo impairment of insulin secretion (Fig. 4). A similar discrepancy between in vivo and in vitro islet function was observed in other animal models of diabetes displaying islet inflammation (46,47).

Our in vitro observations that angiotensin II impairs glucose-stimulated insulin secretion are in line with previous findings in mouse islets showing that treatment

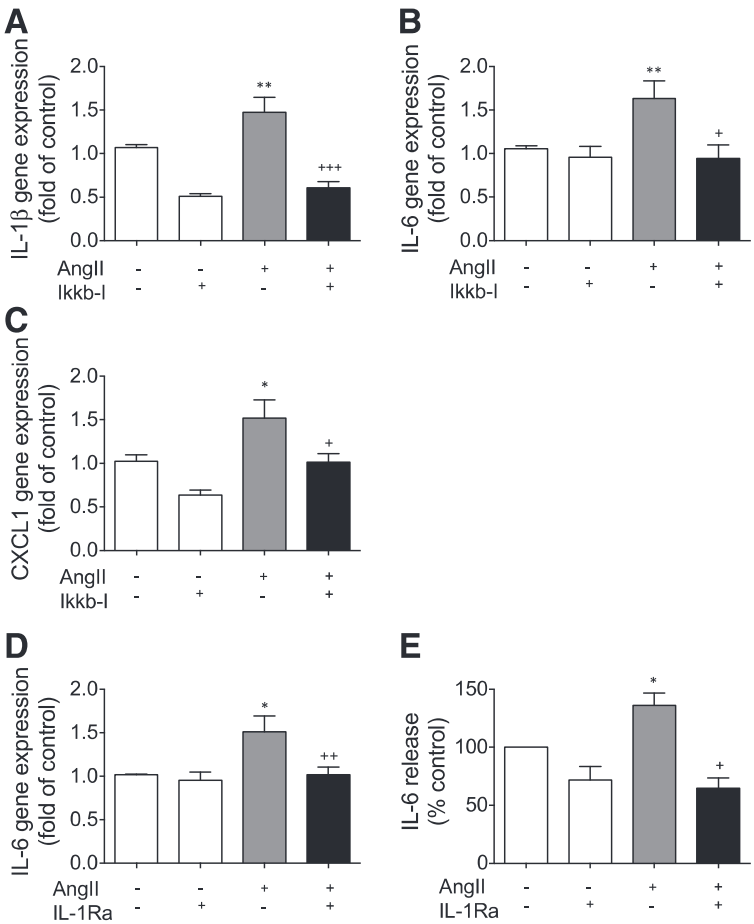

Figure 3—Angiotensin II (AngII)–induced cytokine expression in human islets is mediated by NF-κB and IL-1. Expression of IL-1b (A) and IL-6 (B) genes measured in human islets and CXCL1 (C) in INS-1E cells with and without the IκB-kinase-2 inhibitor SC-514 (IKKb-I) and IL-6 gene expression (D) and protein release (E) with and without IL-1Ra measured in human islets treated with AngII (1 μmol/L) for 4 days. Statistics were performed using one-way ANOVA. Bar graphs show data as mean ± SEM. Data are averages of six (C of three) independent experiments. *P < 0.05, **P < 0.01, control compared with AngII; +P < 0.05, ++P < 0.01, +++P < 0.001, AngII compared with AngII + IKKb-I or AngII + IL-1Ra.

with angiotensin II dose-dependently reduced insulin secretion and synthesis (15). The impairment of the glucose-stimulated secretory function goes together with increased basal insulin secretion in all our in vitro models (Fig. 1). This may be because of the deleterious effects of angiotensin II on β-cells, forcing inadequate continuous release of insulin with consecutive decreased responsiveness to an acute glucose challenge. The data from our study are in apparent contrast to some older studies showing that angiotensin II induces insulin secretion in the absence of glucose stimulation (20). In the presence of large amounts of glucose, however, angiotensin II diminished mitochondrial function and insulin secretion. In MIN6 β-cells, 1 h of angiotensin II treatment potentiated glucose-stimulated insulin secretion (48). Thus acute exposure to angiotensin II promotes insulin secretion, whereas chronic treatment in the presence of elevated glucose concentrations is deleterious. This possibly reflects context-dependent effects of angiotensin II, which may be physiologic or pathologic. In line with this thinking, short-term and low-dose IL-1β stimulates insulin secretion and β-cell survival, whereas prolonged exposure is deleterious (49,50).

Hydralazine has commonly been used to reverse the hypertensive effect of angiotensin II (51). In accordance with these findings, blood pressure measurements in our in vivo experiments revealed vasoconstrictive effects of angiotensin II and the ability of hydralazine to reverse them. Since our aim was to investigate the effects of angiotensin II independent of vasoconstriction, in vivo experiments with animals that received angiotensin II together with hydralazine and the respective controls were performed. Mice treated with angiotensin II and hydralazine exhibited lower body weights compared with saline-treated animals, as previously described (52,53). Despite the lower body weight, the animals

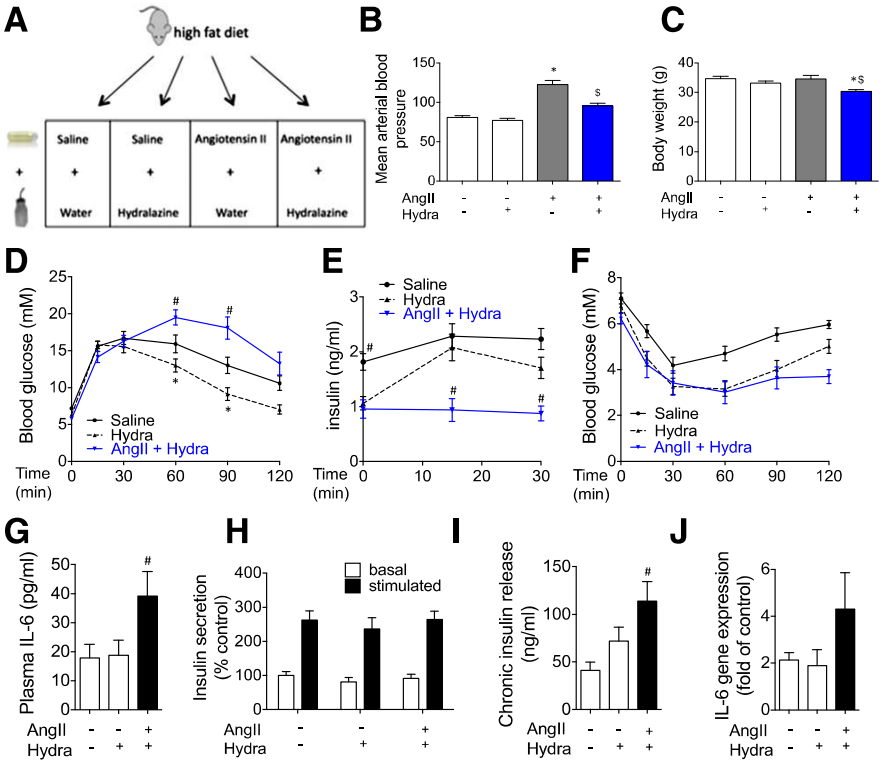

Figure 4—Angiotensin II (AngII) treatment impairs glucose tolerance in vivo. A: Study design: Mice were fed an HFD for 12 weeks and then randomized in four groups of treatment for an additional 4 weeks, as follows: saline, 250 mg/L hydralazine (Hydra), 1 μg/kg/min AngII, or the same doses of AngII and Hydra together. Mean arterial blood pressure measured for 3–5 days (three to eight mice per group; B); mean body weight (14–16 mice per group; C); blood glucose (D) and insulin concentrations (E) during intraperitoneal glucose tolerance tests (16–19 mice per group) and insulin tolerance tests (six or seven mice per group; F) at the end of treatment. G: Plasma IL-6 measurements (14–18 mice per group). Ex vivo glucose-stimulated insulin secretion (two experiments, 10–12 mice per group; H), chronic insulin release (two experiments, 8–12 mice per group; I), and IL-6 gene expression (two experiments, 6–9 mice per group; J) in isolated islets. Statistics were performed using one-way ANOVA. Bar graphs show data as mean ± SEM. Data are averages of three independent experiments (if nothing else mentioned). *P < 0.05 vs. control; $P < 0.05 vs. AngII; #P < 0.05 vs. Hydra.

exhibited impaired glucose tolerance. Both lower body weight and glucose intolerance can be explained by a lack of insulin. This strengthens the assumption that angiotensin II impairs insulin secretion, which was not always observed when hypertension was not corrected (22,48).

Experiments with dispersed human islets revealed an increase in β-cell apoptosis after treatment with angiotensin II. Because cultured human islets grow on multiple layers, which makes the identification of β-cells difficult, whole-islet stainings were limited by technical issues. Although we acknowledge that single β-cells may behave differently than intact islets, at least we are confident that the apoptotic process occurred in β-cells. Corroboratively, we see similar proapoptotic effects of angiotensin II in the β-cell line INS-1E.

Angiotensin II induced proinflammatory cytokines in human islets, including IL-6, IL-1β, IL-8, and MCP-1, reflecting a proinflammatory state in general. We focused on IL-6 as an easily detectable marker of inflammation, not meaning that it is causal for all the effects. Angiotensin II–induced inflammation does not seem to be species dependent since mouse islets showed similar results. Moreover, our data with the pure β-cell line INS-1E indicate that there is a direct proinflammatory effect of angiotensin II on β-cells, although cell lines express a much smaller variety of cytokines.

Cytokine induction seems to be mediated via NF-κB because treatment with IκB-kinase-2 inhibitor fully prevented the angiotensin II–induced upregulation of IL-1β and IL-6 in human islets and CXCL1 in INS-1E cells.

Importantly, by using IL-1Ra, we show that the angiotensin II–mediated induction of IL-6 in human islets depends on IL-1β signaling. IL-1β is a master proinflammatory mediator involved in the development of type 2 diabetes (26); therefore its inhibition is currently in clinical development for the treatment of diabetes. In this study we show that IL-1β also mediates the deleterious effects of angiotensin II on insulin secretion and glucose homeostasis, adding to the rationale for the use of IL-1 antagonism in the treatment of the metabolic syndrome.

Taken together, our results show that chronically elevated angiotensin II concentrations induce β-cell

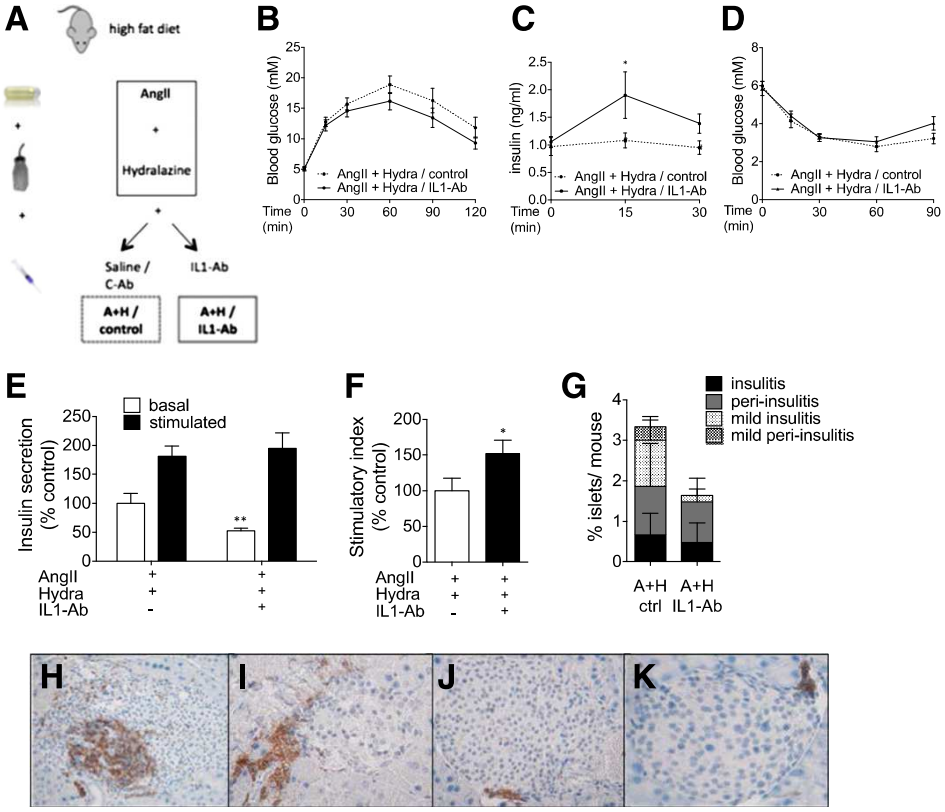

Figure 5—IL-1β antagonism protects from the deleterious effects of angiotensin II (AngII). A: Mice were fed an HFD for 12 weeks and then treated for 4 weeks with 1 μg/kg/min AngII and 250 mg/L hydralazine (Hydra) and injected subcutaneously once a week with either anti–IL-1β antibodies or saline or nonspecific antibodies (control group). Blood glucose (B) and insulin (C) concentrations were measured during intraperitoneal glucose tolerance tests and blood glucose during insulin tolerance tests (D). E: Ex vivo glucose-stimulated insulin secretion. F: Corresponding stimulatory index. G: Percentage of insulitis in the treatment groups. Representative immunohistochemical stainings of $CD45^+$ cells (brown) of pancreatic tissue sections for insulitis (H), peri-insulitis (I), mild insulitis (J), and mild peri-insulitis (K). Statistics were performed using the Student t test. Bar graphs show data as mean ± SEM. Data (B–D) are averages of eight animals per group; data in E and F are from four mice in both groups. *P < 0.05, **P < 0.01, basal AngII/control vs. basal AngII/IL-1 antibodies.

dysfunction in vitro and in vivo, independently of its effects on blood pressure. This effect seems to be mediated by a proinflammatory response via the IL-1β/NF-κB pathway. Therefore, some of the protective effects of ACE inhibitors observed in patients with prediabetes and diabetes could be due to the prevention of angiotensin II–induced islet inflammation.

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Duality of Interest. M.Y.D. is listed as the inventor on a patent (W06709) filed in 2003 for the use of an IL-1 receptor antagonist for the treatment of, or prophylaxis against, type 2 diabetes. No other conflicts of interest relevant to this article have been reported.

Author Contributions. N.S.S., A.W.J., M.B.-S., and M.Y.D. designed the study. N.S.S., C.T., Y.P., and K.K. performed and analyzed experiments. N.S.S., C.T., M.B.-S., and M.Y.D. wrote the manuscript. E.D., S.T., and K.T. helped with the experiments. B.B., F.P., and J.K.-C. isolated human islets. M.Y.D. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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3.2 Unpublished data

3.2.1 Angiotensin II-induced glucose intolerance is independent of GLP-1 *in vivo*

Since the strong *in vivo* inhibitory effects of chronic Ang II treatment on insulin secretion were lost *ex vivo* after islet isolation, we speculated that the inhibition of gut-derived incretins, which predominantly boost insulin secretion after food intake, might be involved in the Ang II effects *in vivo* but not *ex vivo*. Therefore, we treated mice with hydralazine (250 mg/l) in the drinking water to avoid vasoconstriction and with 100 µg Ang II for 4-8 days intraperitoneally every day. At the last treatment day we performed oral glucose tolerance tests to stimulate the GLP-1 secretion and to test the hypothesis of Ang II being an incretin inhibitor. Glucose or saline were injected at time point 0 and blood glucose was measured at time points 0, 10 and 30 min. Blood for measuring active GLP-1 in the plasma was taken at time point 10 min and was supplemented with EDTA and 100 µM dipeptidylpeptidase-IV inhibitor (Ile-Pro-Ile, Sigma, 422 Switzerland) to avoid rapid GLP-1 degradation. Chronic Ang II treatment compared to the saline treated group impairs glucose tolerance when glucose is given orally (Fig. 12A solid lines); however, plasma insulin levels at time point 10 min are unchanged (data not shown). Active GLP-1 levels are elevated at time point 10 min after orally administered glucose but there are no significant differences between Ang II-treated and control mice (Fig. 12B). If anything, GLP-1 levels tend to be higher in the Ang II-treated mice compared to the control.

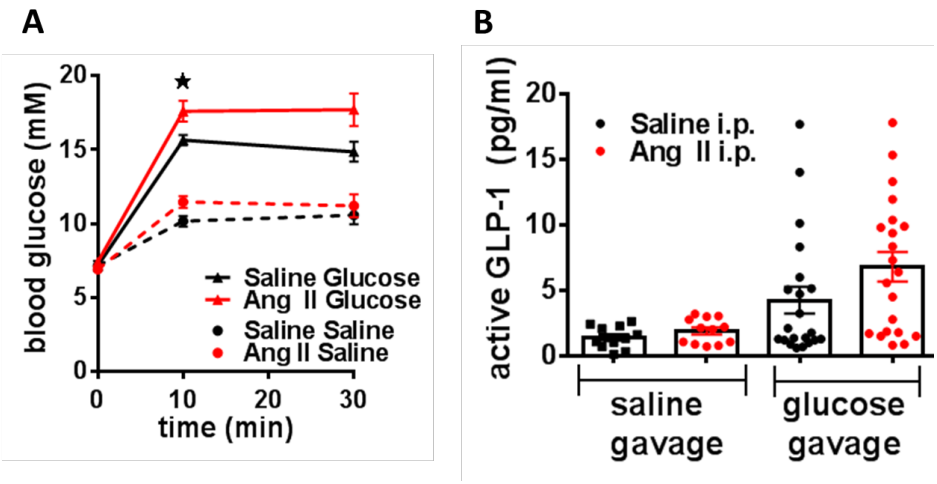

Figure 12. Angiotensin II-induced glucose intolerance is independent of GLP-1 *in vivo*. C57Bl/6 mice received hydralazine (250 mg/l) in the drinking water to avoid vasoconstriction and were treated intraperitoneally (i.p.) with saline (black) or Ang II (red) every day for 4-8 days. **(A)** Oral glucose tolerance tests with saline (dashed lines) or glucose gavages (solid lines). Blood glucose was measured at time points 0, 10 and 30 min. **(B)** Active GLP-1 levels (Mesoscale Discovery) were measured in the plasma at time points 10 min after acute saline or glucose gavages. Blood was directly supplemented with EDTA and a GLP-1 stabilizer (dipeptidylpeptidase-IV inhibitor). Statistics were performed using the Student *t* test. Bar graphs are expressed as mean ± SEM. Glucose data are averages of 3 experiments with a total of 19-20 mice per group. *p < 0.05 Ang II + glucose to saline + glucose.

4 Role of Sirtuin 1 in the development of diabetes

4.1 Manuscript No. 1 - Sirtuin 1 mutation L107P and the development of type 1 diabetes

4.1.1 Abstract

Type 1 diabetes (T1D) is a multifactorial chronic disease characterized by autoimmune reactions against pancreatic β-cells with metabolic dysfunction involving destruction of β-cells and insulin deficiency. Sirtuin 1 (SIRT1) is an NAD^+ dependent class III histone and protein deacetylase, implicated in various cellular processes ranging from the regulation of metabolism, longevity and general stress resistance to inflammation and immune response. However, the influence of SIRT1 activation on metabolism and immunity is unclear. Recently, a point mutation in the *Sirt1* gene, which causes an amino acid exchange from leucine to proline (L107P), was characterized in several members of a family with T1D and colitis. Based on the association of the familial SIRT1 mutation L107P with autoimmune diseases, we investigated the role of SIRT1 in the development of autoimmunity and T1D. Therefore, we generated rat INS-1E cells ectopically overexpressing the human mutated SIRT1 and a knock-in SIRT1 mouse model carrying the corresponding mouse mutation L102P. Besides elevated levels of reactive oxygen species, the modified INS-1E β-cells exhibited glucose-stimulated insulin secretion defects. However, our *in vivo* data with the SIRT1 L102P knock-in mice revealed new perspectives to the SIRT1 mutation and its effects on immune responses and diabetes. The mutation of SIRT1 enhanced insulin expression and secretion, induced islet inflammation and an autoimmune-like phenotype along with a reduced number of regulatory T-cells. We propose that these effects are due to a constitutive overactivation of SIRT1, possibly leading to metabolic deterioration with increasing age or additional triggers. Thus, SIRT1 has complex functions in the regulation of metabolic and immune reactions. Inhibition of SIRT1 may have therapeutic value in the development of T1D and other autoimmune diseases.

4.1.2 Introduction

Type 1 diabetes (T1D) is a multifactorial chronic disease with both genetic and environmental causes but the exact mechanisms of the initiation and progression are not fully understood. The disease is characterized by autoimmune reactions against pancreatic β-cells with metabolic dysfunction involving destruction of β-cells and insulin deficiency. There is increasing evidence that dysfunctional suppressive capacity of regulatory T-cells (Tregs) contributes to imbalanced autoreactive T-cells and failure in maintaining self-tolerance [240], [228]. In the last years, abundant research was done on sirtuins, a family of proteins that are known to be involved in the regulation of numerous cellular processes leading to metabolic and immunological homeostasis [121]. Sirtuin 1 (SIRT1) is an NAD^+ dependent class III histone and protein deacetylase, which has various functions ranging from the regulation of metabolism, circadian rhythm, cell cycle, longevity and general stress resistance to inflammation and immune response. SIRT1 acts as energy sensor and its overexpression enhances insulin secretion [167], [168]. The lack of SIRT1 has been linked to the development of metabolic diseases, mainly type 2 diabetes (T2D), due to maladaptation to metabolic stress [172], [173], [174]. However, the influence of SIRT1 activation on metabolism and other physiological and pathophysiological processes is not fully understood.

Recently, a point mutation in the *sirtuin 1* gene, which causes an amino acid exchange from leucine to proline (L107P), was characterized in several members of a family with autoimmune diseases [215]. The mutation lies outside of the catalytic core but may influence protein-protein interactions. Four patients displayed typical features of T1D including autoantibodies against β-cell antigens, hyperglycemia and rapid dependence on insulin, whereas one family member developed ulcerative colitis.

SIRT1 is highly expressed in dendritic cells, activated and anergic T-cells and various other immune cells [217], [218] but not much has been reported on SIRT1 and its role in the innate and adaptive immune response along with autoimmunity. There is evidence that SIRT1 is involved in T-cell differentiation and tolerance by targeting transcription factors including forkhead box p3 (Foxp3), activator protein-1 (AP-1), nuclear factor-kappa B (NF-κB) and retinoic acid receptor-related orphan receptor-γt (RORγt).

SIRT1 inhibits transcription and production of IL-2, which is a critical factor for the development of functional effector T-cells and which is able to reverse T-cell anergy and tolerance [218], [224]. In parallel, IL-2 is a central regulator for the suppressive function of regulatory T-cells and therefore immune tolerance [225]. In early studies, cyclosporine was used as immunotherapeutical treatment against IL-2-induced autoreactive T-cell activation in T1D patients but the beneficial effects were only temporary [226]. Interestingly, the lack of IL-2 has been shown to play a crucial role in the development of T1D [227] and therapies with low-dose IL-2 treatment to boost or restore regulatory T-cells protected from the development of T1D in NOD mice and in clinical studies [228], [229]. The transcription factor Foxp3, which is a key factor for the development and function of Tregs, is deacetylated by SIRT1 leading to proteasomal degradation [23], whereas blockage of SIRT1 improved Treg suppressive function *in vitro* and *in vivo* [232]. It has been convincingly demonstrated in a recent study that SIRT1 deacetylates the transcription factor RORγt, which regulates the differentiation of Th17 effector cells, and thereby enhances the Th17/Treg ratio and the susceptibility to autoimmune diseases [233]. Additionally, inhibition of histone deacetylases (HDACs) was suggested to improve Treg function in autoimmune diseases including colitis [234], [235]. There is evidence that the deacetylase SIRT1 also influences the development of colitis since intestinal deletion of SIRT1 protected from colitis and colitis-induced colorectal cancer by re-arranging the gut microbiome [236]. Contradictory observations were made with whole body Sirt1 -/- mice, which develop an autoimmune-like phenotype with increased anti-nuclear antigen antibodies [238]; however, they are developmentally affected, infertile and have a small body size.

Based on the association of the familial SIRT1 mutation L107P with autoimmune diseases, we wanted to investigate the underlying mechanisms of SIRT1-mediated effects in the development of autoimmunity and T1D. Therefore, we generated rat INS-1E cells ectopically overexpressing the human mutated SIRT1 and a knock-in SIRT1 mouse model carrying the corresponding mouse mutation L102P. Our results support a new perspective for understanding the complex functions of SIRT1 as an essential player in immune responses.

4.1.3 Methods

4.1.3.1 INS-1E cell lines

INS-1E cells ([241], kindly provided by C. Wollheim) were stably transduced with pMSCV-SIRT1-Flag plasmids containing human wildtype SIRT1 ("WT") or mutated SIRT1 L107P ("L107P") or an empty plasmid ("empty") as control using murine retroviruses (kindly provided by Basil Hubbard, Harvard Medical School). Human SIRT1 expression was analyzed with TaqMan real-time PCR assays and by Western blotting using anti-human SIRT1 antibodies (H-300, Santa Cruz Biotechnology) and anti-FLAG antibodies (A9594, SIGMA). INS-1E cell lines were cultured in INS-1E medium: RPMI medium containing 11.1 mM glucose, 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamax, 10% FCS, 10 mM HEPES, 1 mM sodium pyruvate, and 50 mM 2-mercaptoethanol and 10 µg/ml puromycin. For glucose-stimulated insulin secretion assays and RNA isolation, 200'000 cells/well were seeded in 24-well plates in quadruplicates 2 days before the experiment.

4.1.3.2 Oxygen consumption assay

Seahorse Bioscience technology was used to test the mitochondrial function of INS-1E cells. 250'000 cells/well were plated on a poly-D-lysine pre-coated XF24 microplate (Seahorse Bioscience) and incubated in normal INS-1E medium for 6 h at 37°C and 5% CO_2. In the evening, the INS-1E medium was discarded and changed to starvation medium (RPMI-1640 medium supplemented with 2.8 mM glucose and without 2-mercaptoethanol) for 18 h. The next day, the starvation medium was replaced by assay medium for 2 h at 37°C in air: 1:10 dilution of 10 times concentrated seahorse Krebs Ringer buffer (114 mM sodium chloride, 4.7 mM potassium chloride, 2.5 mM calcium chloride dihydrate, 1.2 mM mono potassium sulfate, 1.2 mM magnesium sulfate heptahydrate), 20 mM HEPES and 0.2% bovine serum albumin (pH 7.2) containing 2.8 mM glucose. To test the reaction to high glucose conditions, ATP turnover, maximal respiratory capacity and non-mitochondrial respiration of the islets, 16.7 mM glucose, 1 µM oligomycin, 7.7 µM of the uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), 1 µM rotenone and 1.7 µM myxothiazol were injected successively and oxygen consumption rates were determined.

4.1.3.3 Transmission electron microscopy

For transmission electron microscopy, 5 Mio INS-1E cells of each group (empty, WT and L107P) were seeded in 75 cm^2 flasks and incubated overnight at 37°C and 5% CO_2. The next day, cells were starved by changing the medium to PBS supplemented with 1 mM sodium pyruvate for 5 h. Then, cells were harvested and processed for electron microscopy by fixation with Karnovsky's paraformaldehyde-glutaraldehyde [242] and 1% osmiumtetroxid, dehydratation with ethanol and embedding with Epon 812 (FLUKA, 45345). 60 nm sections were cut with a microtome (Ultracut E, Leica) and imaging was performed with a Morgagni FEI microscope. Length and width at three different positions of the mitochondria was measured and blinded analyzes were done evaluating nine pictures with several mitochondria per condition. Aspect ratio of mitochondria was defined as length/average of width.

4.1.3.4 MitoTracker staining for the determination of mitochondrial mass and membrane potential

Mitchondrial mass and membrane potential of INS-1E cells were measured using MitoTracker Staining Green and Red, respectively (Life Technologies, M-7514 and M22425). Green is a green-fluorescent dye, which stains mitochondria in live cells and Red stains mitochondrial membrane potential dependently. 500'000 cells/well were seeded in a 24-well plate in INS-1E medium the day prior to the experiment. The next day, cells were stained either with 100 mM MitoTracker Green or 250 mM MitoTracker Red for 30 min at 37°C and 5% CO_2. After two washing steps with PBS, cells were resuspended in 1 ml PBS and fluorescence levels were determined with a flow cytometer (FACS Calibur, BD Biosciences).

4.1.3.5 ATP measurements

ATP levels were measured using the bioluminescent ADP/ATP ratio assay kit from abcam (ab65313). Luciferase catalyzes the conversion of ATP and luciferin to light that can be measured in a luminometer. 1000 INS-1E cells/well were seeded in a cell culture 96-well-plate and two days later supernatants were transferred into a white walled 96-well-plate. Experimental procedures were done following the manufacturer´s guidelines and ATP levels were detected in a luminometer.

4.1.3.6 Protein determination by BCA assay

For the quantification of total protein in INS-1E cells, the Pierce BCA protein assay kit was used according to the manufacturer's guidelines (Thermo Scientific, 23225).

4.1.3.7 Detection of reactive oxygen species

The detection of reactive oxygen species (ROS) including hydrogen peroxide, hydroxyl radicals and peroxynitrite was done by using 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; C6827, Invitrogen). This chloromethyl derivative of dichlorofluorescein (DCF) diffuses passively into cells and is retained after cleavage of its acetate groups by intracellular esterases. The non-fluorescent CM-H_2DCFDA turns into the highly fluorescent DCF upon oxidation by ROS and can be detected with a flow cytometer. 500'000 INS-1E cells/well of all three transfected cell lines (empty, WT, L107P) were seeded in a 24-well plate. Two days later, the cells were trypsinized and resuspended in DCF staining medium: Phenol red free DMEM (Gibco A14430-01) with 11.1 mM glucose, 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamax, 10 mM HEPES, 1 mM sodium pyruvate and 10 µg/ml puromycin. The cells were stained with 10 µM CM-H2DCFDA for 20 min at 37°C in the dark. After washing twice with cold PBS, cells were resuspended in 1 ml cold PBS and analyzed with a flow cytometer (FACS Calibur, BD Biosciences).

4.1.3.8 Animals

Heterozygous knock-in SIRT1 L102P animals were generated by GenOway via homologues recombination in embryonic stem cells. The point mutation (in mice called L102P) was inserted into exon 1a of the *Sirt1* gene, with a targeting vector containing regions homolog to the genomic *Sirt1* sequences and a neomycin selection cassette, thereby disrupting the murine *Sirt1* gene function (Fig. 1). The mutated gene is expressed under the control of the endogenous SIRT1 promoter.

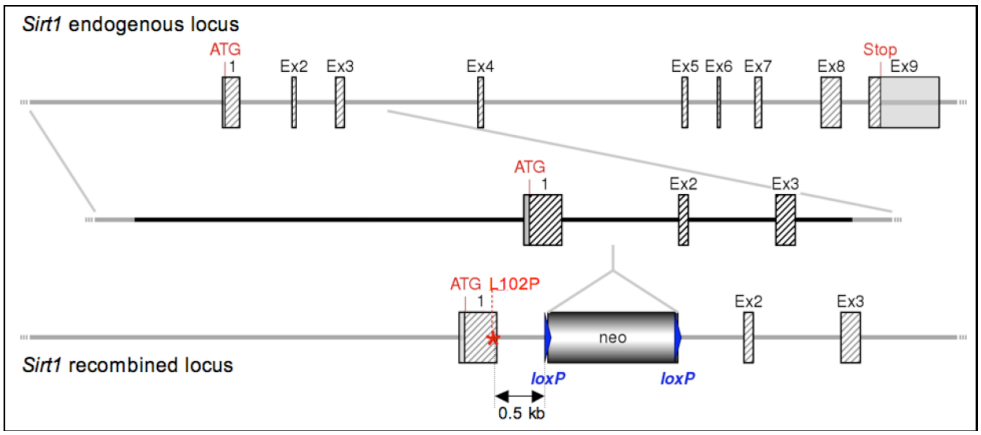

Figure 1. Targeting strategy of L102P SIRT1 knock-in mice. The point mutation L102P was inserted into exon 1a of the *Sirt1* gene via homologues recombination of embryonic stem cells and is expressed under the control of the endogenous *Sirt1* promoter. A neomycin cassette flanked by loxP sites acted as positive selection marker.

The mice were backcrossed on a clean C57Bl/6 J background. Heterozygous animal breeding was performed to obtain wildtype ("WT"), heterozygous ("het") and homozygous ("homo") littermates. Separate wildtype and homozygous breedings were started to obtain more animals with the same age at the same week, leading to a mixture of littermates and non-littermates in single experiments. High-fat diet feeding (D12331; Research Diets, New Brunswick, NJ) was started at the age of 5 weeks and cages and food were changed once a week. All animals were housed in a temperature-controlled room with a 12 h light/12 h dark cycle and were allowed free access to food and water. Mice were sacrificed, blood was obtained by heart puncture and plasma was collected for later measurements of IL-2 protein (Mesoscale Discovery). All animals were conducted according to the swiss veterinary law and to institutional guidelines.

4.1.3.8 Glucose and insulin tolerance tests

For intraperitoneal glucose tolerance tests, mice were fasted for 6 h in the morning and injected intraperitoneally with 2 g glucose/kg body weight. Blood glucose was measured at time points 0, 15, 30, 60, 90, and 120 min using a glucometer (Freestyle; Abbott Diabetes Care Inc., Alameda, CA) and blood was collected at 0, 15 and 30 min for measurement of plasma insulin concentrations using mouse insulin assays (Mesoscale Discovery). For intraperitoneal insulin tolerance tests, mice were fasted 3 h in the morning before

administration of 1 or 2 units/kg insulin (Novo Nordisk, Bagsvaerd, Denmark), and blood glucose was measured at time points 0, 15, 30, 60, and 90 min.

4.1.3.9 Islet isolation

For mouse islet isolation, the pancreas was perfused with HBSS containing 1.4 mg/ml collagenase (Worthington, Lakewood, NJ), 10mg/ml DNase (1:3000, Roche, Cat. No. 11284932001) and 0.5% w/v BSA (Merck, 1120180100) and digested for 30 min at 37°C and 5% CO_2, followed by washing steps and filtration through 70 and 500 µm cell strainers. Islet culture medium consisted of RPMI-1640 medium containing 11.1 mM glucose, 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamax, 50 mg/ml gentamicin, 1:1000 Fungizone (Gibco), and 10% FCS. Islets were cultured on extracellular-matrix coated 24-well-plates (Novamed Ltd, Jerusalem, Israel) for 36 h prior to treatment with IL-1β and subsequent RNA isolation or for glucose-stimulated insulin secretion experiments.

4.1.3.9.1 Glucose-stimulated insulin secretion

For *in vitro* or *ex vivo* glucose-stimulated insulin secretion experiments, 25 islets or 200'000 INS-1E cells/well were seeded in quadruplicates. At the next day, INS-1E cells were preincubated in INS-1E medium containing 2.8 mM glucose overnight. At day 2 after plating, supernatants of INS-1E cells or islets were collected and stored at -20°C (chronic insulin release). Cells were incubated for 30 min in modified Krebs-Ringer bicarbonate buffer (mKRBB = 115 mM sodium chloride, 4.7 mM potassium chloride, 2.6 mM calcium chloride dihydrate, 1.2 mM mono potassium sulfate, 1.2 mM magnesium sulfate heptahydrate, 10 mM HEPES and 0.5% bovine serum albumin [pH 7.4]) containing 2.8 mM glucose. Supernatants were discarded and cells or islets were incubated in new mKRBB supplemented with 2.8 mM glucose for 1 h (basal insulin release), followed by 1 h in mKRBB containing 16.7 mM glucose (stimulated insulin release). Islets or INS-1E cells were extracted with 0.18 N hydrogen chloride in 70% ethanol (HCl/EtOH) for 2 h in room temperature to determine insulin content. Insulin concentrations were measured using rat insulin ultrasensitive ELISA (Mercodia, Uppsala, Sweden) or mouse/rat insulin kit (Mesoscale Discovery, Rockville, MD). Stimulatory index was determined as the ratio of stimulated to basal insulin release.

4.1.3.9.2 RNA isolation and quantitative PCR

80 islets isolated as described above were plated on extracellular matrix-coated 24-well-plates for 36 h. Medium was changed and islets were treated with or without 1 ng/ml recombinant mouse IL-1β (R&D Systems, 401-ML) for 24 h. For total RNA extraction the Nucleo Spin RNA kit (Macherey-Nagel GmBH, Düren, Germany) was used and the isolated RNA was reverse transcribed using Superscript II (Invitrogen) and random hexamer primers (Microsynth, Balgach, Switzerland). Quantitative PCR was done with the real-time PCR system 7500 (Applied Biosystems) and following TaqMan gene expression assays were used: mouse: β-actin: Mm00607939_s1, Sirt1: Mm01168519_m1, IL-6: Mm00446190_m1, IL-1β: Mm00434228_m1, IL-1Rn: Mm00446185_m1, IL-22R: Mm01192943_m1, IL-2R: Mm01340213_m1, Ins2: Mm00731595_gH, Pdx1: Mm00435565_m1, Foxo1: Mm00490672_m1. Rat: β-actin: Rn00667869_m1, Sirt1: Rn01428096_m1, Cxc1: Rn00578225_m1 and Ucp2: Rn01754856_m1. Human: 18S: Hs99999901_s1 and Sirt1: Hs01009006_m1.

4.1.3.9.3 Peritoneal macrophages

After sacrifice of the mice peritoneal macrophages were isolated by infusion of the peritoneum with 10 ml PBS + 1% FCS and filtering the lavage through a 70 µm filter (BD Biosciences). 200'000 cells/well were cultured in 48-well plates in macrophage medium (RPMI-1640 medium containing 11.1 mM glucose, 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamax, 50 mg/ml gentamicin, 1:1000 Fungizone (Gibco), and 10% FCS) and incubated overnight. Macrophages were activated with 100 ng/ml LPS + 10 ng/ml recombinant mouse IFNy for 5 h with or without adding 5 mM ATP for the last 30 min. Supernatants were collected and used for measurements of IL-10 protein (Mesoscale Discovery).

4.1.3.9.4 Flow cytometry for immune cell phenotyping

After sacrifice of the mice, inguinal lymph node cells were collected by passing them through a 70 µm filter (BD Falcon). Cell suspensions were resuspended in isolation buffer (PBS + 2% FCS + 5 mM EDTA) and Fc receptor blocked with anti-mouse CD16/CD32 antibodies. Surface

staining was performed for 30 min at 4°C with anti-CD3e (clone 145-2C11), anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7), anti-CD25 (clone PC61.5) and anti-B220 (clone RA3-6B2) or anti-CD19 (clone eBio1D3) antibodies or appropriate isotype controls; all antibodies were purchased from eBioscience or Biolegend. For intracellular staining, cells were fixed and permeabilized with the Foxp3 staining buffer set (eBioscience Cat. No. 00-5523-00), and incubated for 30 min at room temperature with anti-Foxp3 antibodies (clone MF-14). Stained cells were washed twice with FACS buffer (PBS + 0.5% BSA + 5 mM EDTA) prior to analysis on a BD Accuri C6 cytometer (BD Biosciences) using FACS Diva software (BD Biosciences). Data were analyzed using Flow Jo 9.4 software (Tree Star).

4.1.3.9.5 Statistics

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software). Data are presented as mean ± SEM and were analyzed using one-way ANOVA. Differences were considered statistically significant when $p < 0.05$.

4.1.4 Results

4.1.4.1 L107P INS1E cells are more prone to inflammation and have impaired insulin secretion compared to cells overexpressing wildtype SIRT1

In a previous study, transduced mouse insulinoma MIN6 cells were generated to develop a cell model for a familial *Sirt1* mutation leading to autoimmune diabetes and colitis [215]. In the present study, we transduced rat insulinoma INS-1E cells with the same virus stocks that were used for the MIN6 cells. Three INS-1E cell lines were created by puromycin selection: an empty vector control (“empty”), a human SIRT1 overexpressing line (“WT”) and a cell line overexpressing the human mutated SIRT1 L107P (“L107P”). All three cell lines were stably transduced and the ectopic expression of human SIRT1 protein in the WT and L107P cell line was comparable as detected with anti-human SIRT1 (Fig. 2A) and anti-FLAG antibodies and the β-actin control (Fig. 2B).

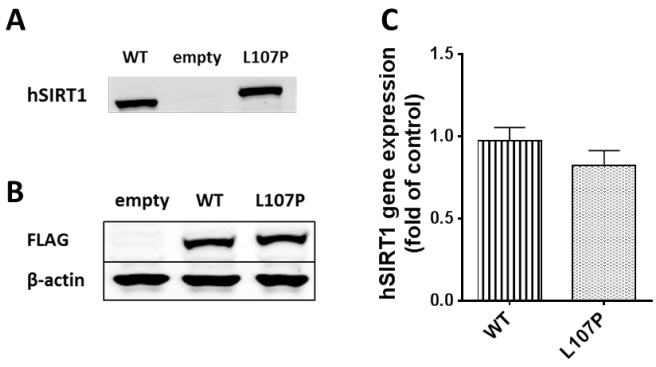

Figure 2. Transduction of INS-1E cells resulted in equal levels of human SIRT1 protein in L107P cells compared to wildtype SIRT1 overexpressing cells. (A) Representative pictures for protein levels of human SIRT1 in transduced INS-1E cells (WT, empty and L107P) stained with anti-human SIRT1 antibodies from Santa Cruz or **(B)** with anti-FLAG antibodies (A9594, SIGMA). **(C)** Gene expression levels of human *Sirt1* are slightly lower in transduced L107P INS-1E cells compared to the wildtype control. Bar graphs are expressed as mean ± SEM. Data (C) are averages of four independent experiments.

Since decreased anti-inflammatory activities of mouse insulinoma MIN6 cells overexpressing the human mutated L107P SIRT1 compared to the WT SIRT1 were shown [215], we examined if we see similar effects in the rat β-cell line INS-1E after transduction. Upon treatment with 1 ng/ml IL-1β for 24 h, the mutated INS-1E cells (L107P) reacted with a slightly lower reduction of *Cxcl1* gene expression levels than the wildtype SIRT1 cell line compared to the empty control (Fig. 3A). Furthermore, the induction of reactive oxygen species (ROS), detected by CM-H2DCFDA staining, was elevated in untreated L107P INS-1E cells compared to both controls (Fig. 3B). The overexpression of WT SIRT1 did not change the levels of ROS compared to the empty control (Fig. 3B). Glucose-stimulated insulin secretion experiments revealed lower insulin secretion capacity of the L107P cells compared to the controls (Fig. 3C). Again, overexpression of WT SIRT1 did not change the insulin secretion of INS-1E cells compared to the empty control (Fig. 3C). Chronic insulin release into the culture medium and islet insulin content were comparable in the three cell lines (data not shown). To test if elevated uncoupling contributed to the diminished insulin secretion of L107P INS-1E cells, we measured the basal gene expression levels of *Ucp2* and did not reveal significant differences in the three cell lines (data not shown).

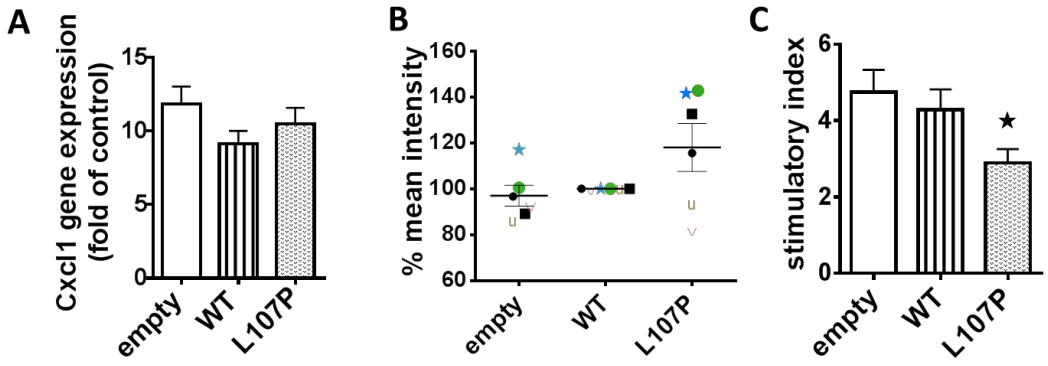

Figure 3. L107P INS1E cells are more prone to inflammation and have insulin secretion defects compared to cells overexpressing wildtype SIRT1. (A) Gene expression of *Cxcl1* in transduced INS1-E cells (empty, WT and L107P), expressed relative to 18S. Cells were treated with 1 ng/ml IL-1β for 24 h. **(B)** Measurements of reactive oxygen species in transduced INS1-E cells via CM-H2DCFDA staining and flow cytometry. Each symbol represents one separate experiment and data is normalized to the mean fluorescence intensity of each WT data, expressed as percent. **(C)** Glucose-stimulated insulin secretion assays of transduced INS1-E cells, expressed as stimulatory index (stimulated to basal insulin secretion). Statistics were performed using one-way ANOVA. Bar graphs are expressed as mean ± SEM. Data are averages of four (A), three (B) and five (C) independent experiments. *p<0.05 L107P to empty. CM-H2DCFDA: 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate.

To further understand the lower insulin secretion capacity of the INS-1E L107P cells, we assessed the function and morphology of the mitochondria. First, we performed experiments with the Seahorse technology (Bucher AG) to test oxygen consumption of the INS-1E cell lines. No significant differences in oxygen consumption rates (OCR) were observed between the three overnight starved INS-1E cell lines after injection of 16.7 mM glucose (HG), oligomycin and rotenone plus myxothiazol (rot + myx). The maximal respiratory capacity was mildly increased in both WT and L107P SIRT1 cells compared to the empty control cells, indicated through injection of the uncoupler FCCP (Fig. 4A). However, no differences in oxygen consumption were observed between the WT and L107P SIRT1 overexpressing cells. Second, we measured basal ATP levels of the three INS-1E cell lines to investigate the bioenergetic state of the cells but we did not detect any changes between the cell lines (Fig. 4B). Additionally, mitochondrial mass and membrane potential were examined by MitoTracker Green and Red stainings, respectively (Life Technologies). No significant changes were revealed under basal conditions between the INS-1E cell lines (Fig. 4C+D).

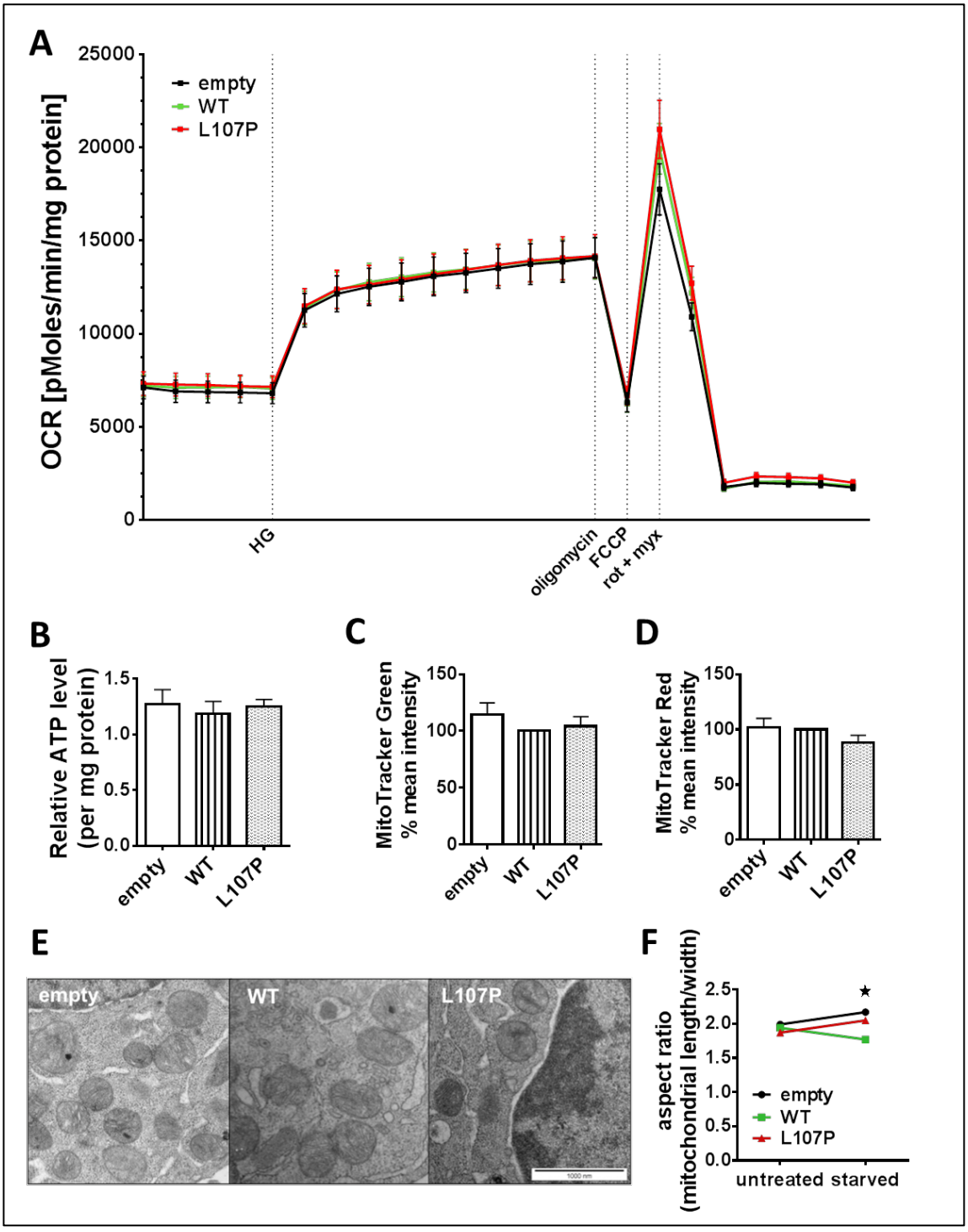

Figure 4. L107P INS1E cells exhibit similar oxygen consumption rates, ATP levels and mitochondrial mass and membrane potential compared to cells overexpressing wildtype SIRT1. (A) Oxygen consumption rates of INS-1E cells (empty, WT, L107P) after 18h starvation in low glucose medium. Successive injections of 16.7 mM glucose (HG), oligomycin, FCCP and rotenone together with myxothiazol (rot+myx). **(B)** ATP levels under basal conditions in the three INS-1E cell lines, expressed per mg protein. Mitochondrial mass was detected by MitoTracker Green **(C)** and mitochondrial membrane potential by MitoTracker Red **(D)** staining in the INS-1E cell lines under basal conditions. Results are normalized to the WT cells and expressed as percent of mean fluorescent intensity. **(E)** Representative transmission electron microscopy pictures of the three cell lines during starvation (5 h PBS with 1 mM sodium pyruvate). **(F)** Aspect ratio is defined as mitochondrial length to width. Statistics were performed using one-way ANOVA. Bar graphs are expressed as mean ± SEM. Data are averages of three (A+B), five (D) and six (C) independent experiments. *$p < 0.05$ WT to empty.

However, the L107P cells tended to have a lower mitochondrial membrane potential compared to the two other cell lines, without reaching significance (Fig. 4D). Third, we characterized the morphology of the mitochondria after starvation by transmission electron microscopy (Fig. 4E). The aspect ratio of the mitochondria, which is defined as the ratio of mitochondrial length to width, was decreased in the WT SIRT1 but not in the L107P SIRT1 overexpressing cells compared to the empty controls during starvation conditions (Fig. 4F).

4.1.4.2 Homozygous L102P SIRT1 mice exhibit mildly improved glucose and insulin tolerance and enhanced β-cell function

To obtain an animal model system reflecting the SIRT1 mutation in the diabetic patients [215], we generated heterozygous and homozygous SIRT1 L102P knock-in mice that carry the corresponding mouse mutation. To metabolically phenotype these mice, we performed glucose and insulin tolerance tests with mice of different sex and age. At the age of 12 and 20 weeks, we saw no differences of glucose or insulin tolerance between WT (green), heterozygous (black) and homozygous (red) knock-in male mice (Fig. 5A-F), despite higher basal and glucose-stimulated plasma insulin levels in the homozygous mice during glucose tolerance tests at 12 weeks of age (Fig. 5B).

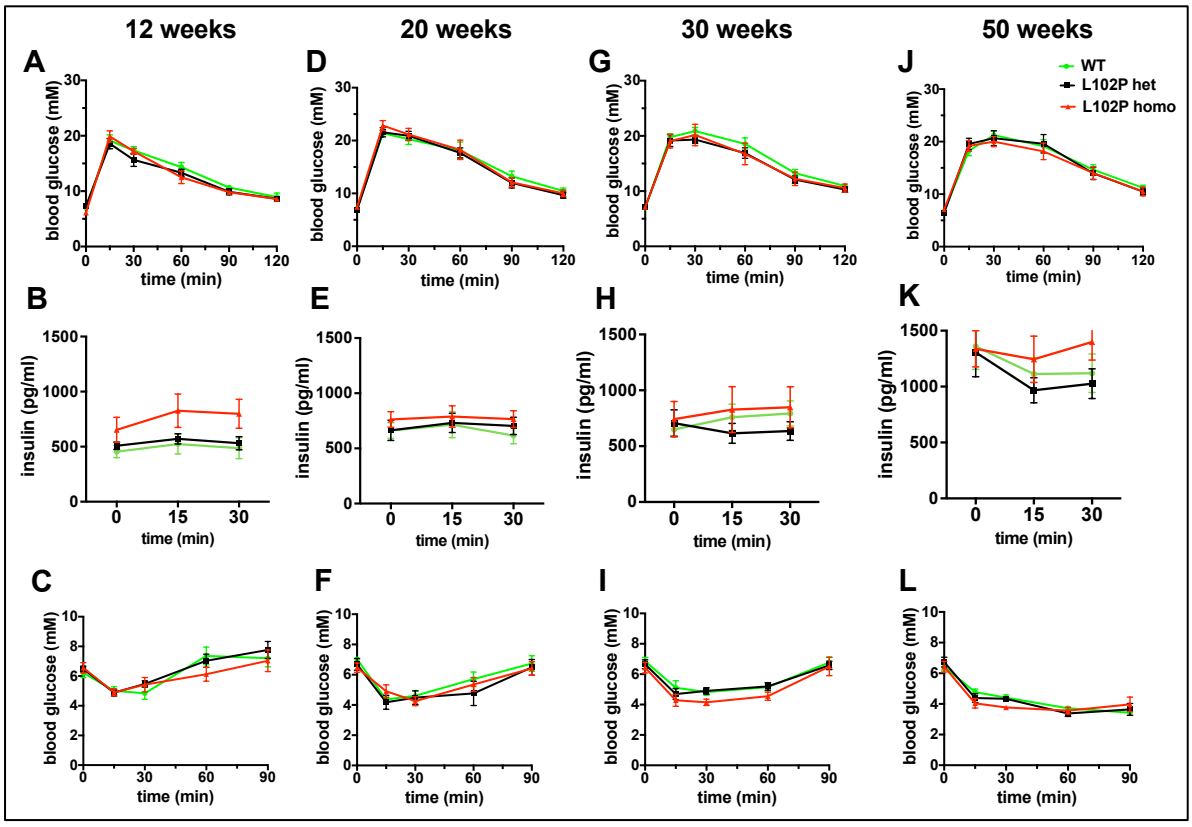

Figure 5. Homozygous L102P SIRT1 male mice exhibit slightly improved glucose and insulin tolerance at the age of 30 weeks compared to wildtype mice when fed a chow diet. Intraperitoneal glucose tolerance tests of the L102P mice (WT green, het black and homo red) at the age of 12 **(A)**, 20 **(D)**, 30 **(G)** and 50 **(J)** weeks and the corresponding insulin measurements at time points 0, 15 and 30 **(B, E, H, K)**. Intraperitoneal insulin tolerance tests at the age of 12 **(C)**, 20 **(F)**, 30 **(I)** and 50 **(L)** weeks. Bar graphs are expressed as mean ± SEM. Data are averages of two independent cohorts with a total of 10 (WT), 12 (het) and 8 (homo) mice. Single mice were excluded when the injection of glucose/ insulin obviously did not work.

A slight improvement of both the glucose and insulin tolerance was observed at the age of 30 weeks in male homozygous knock-in mice compared to the WT mice (Fig. 5G+I and Fig. 6A+B), even if the insulin levels were comparable (Fig. 5H). 50-weeks-old male mice of all strains did not react differently to glucose or insulin (Fig. 5J+L).

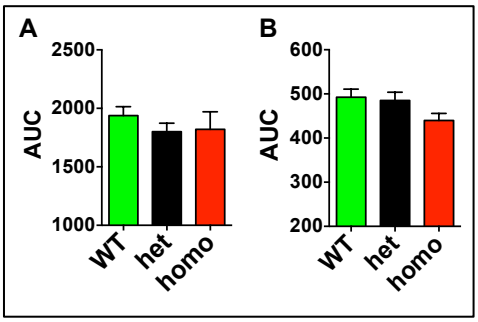

Figure 6. Glucose and insulin tolerance tests of L102P SIRT1 male mice at the age of 30 weeks fed a chow diet. Intraperitoneal glucose **(A)** and insulin **(B)** tolerance tests are expressed as area under the curve (AUC). Bar graphs are expressed as mean ± SEM.

In female mice, insulin tolerance tests at the ages 12, 20, 30 and 50 weeks (Fig. 7C, F, I, L) and glucose tolerance tests at the age of 12 weeks (Fig. 7A) did not reveal differences between the three groups.

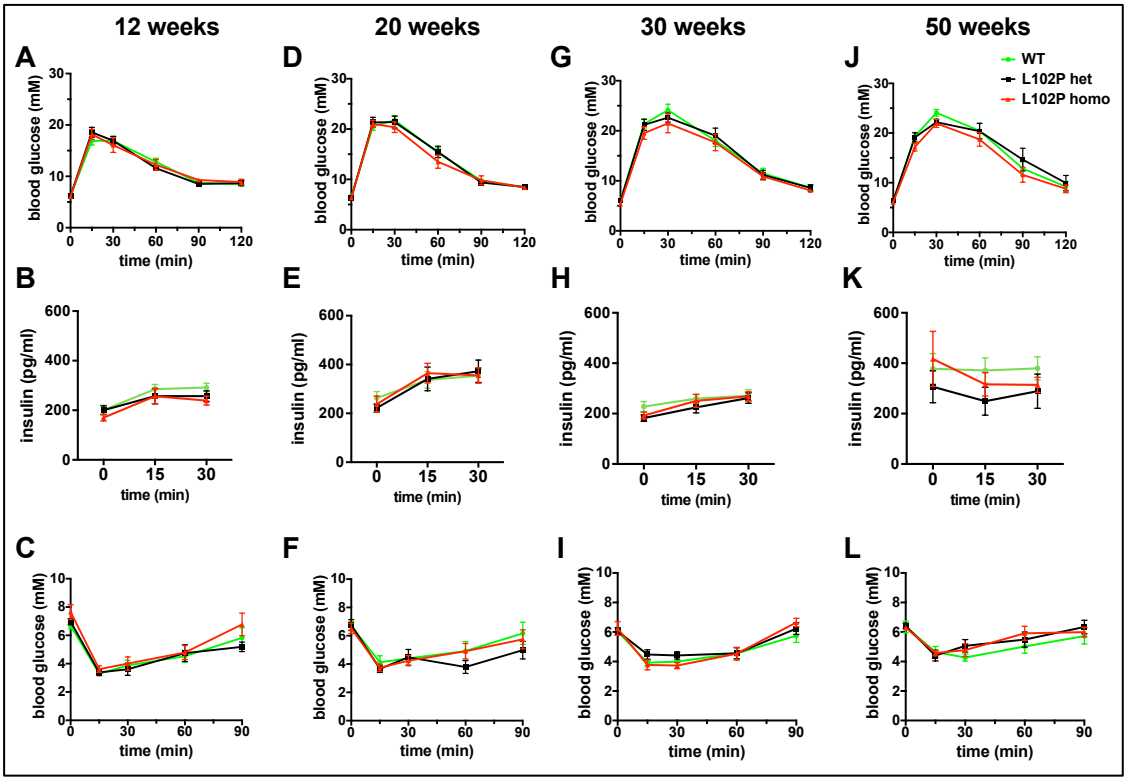

Figure 7. Homozygous L102P SIRT1 female mice exhibit improved glucose and insulin tolerance at the age of 30 and 50 weeks compared to wildtype mice when fed a chow diet. Intraperitoneal (ip) glucose tolerance tests at the age of 12 **(A)**, 20 **(D)**, 30 **(G)** and 50 **(J)** weeks and the corresponding insulin measurements at time points 0, 15 and 30 **(B, E, H, K)**. Ip insulin tolerance tests at the age of 12 **(C)**, 20 **(F)**, 30 **(I)** and 50 **(L)** weeks. Bar graphs are expressed as mean ± SEM. Data are averages of three (two for J, K and L) independent cohorts with a total of 20 (WT), 19 (het) and 15 (homo) mice. Single mice were excluded when the injection of glucose/insulin obviously did not work.

Glucose-stimulated insulin levels did not alter throughout the whole testing at the ages of 12, 20, 30 and 50 weeks (Fig. 7B, E, H, K). However, slight improvements of the glucose tolerance in female homozygous compared to wildtype mice started at the age of 20 weeks and became more pronounced at the ages of 30 and 50 weeks (Fig. 7G, J and Fig. 8A+B), without reaching statistical significance.

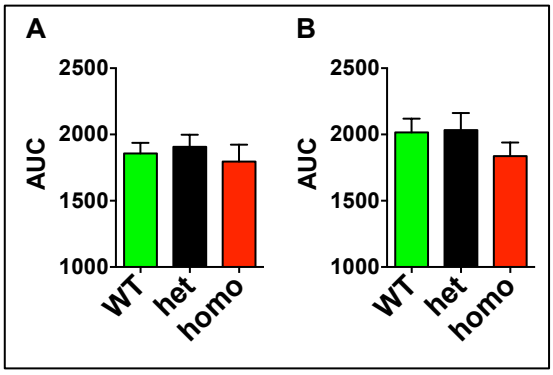

Figure 8. Glucose tolerance tests of L102P SIRT1 female mice at the age of 30 and 50 weeks fed a chow diet. Intraperitoneal glucose tolerance tests at the age of 30 **(A)** and 50 **(B)** weeks are expressed as area under the curve (AUC). Bar graphs are expressed as mean ± SEM.

Furthermore, we challenged the L102P SIRT1 mice with a high-fat diet feeding starting at the age of 5 weeks to see if the previously described effects would be more pronounced or if we could induce the precipitation of a disease. Glucose and insulin tolerance were tested at the same ages as the mice fed a chow diet (Fig. 5+7). At the tested ages of 12, 20 and 30 weeks, male mice fed a high-fat diet did not reveal differences in glucose or insulin tolerance (Fig. 9A-I). However, 30-week-old male homozygous mice tended to have impaired glucose tolerance compared to the wildtype mice (Fig. 9G).

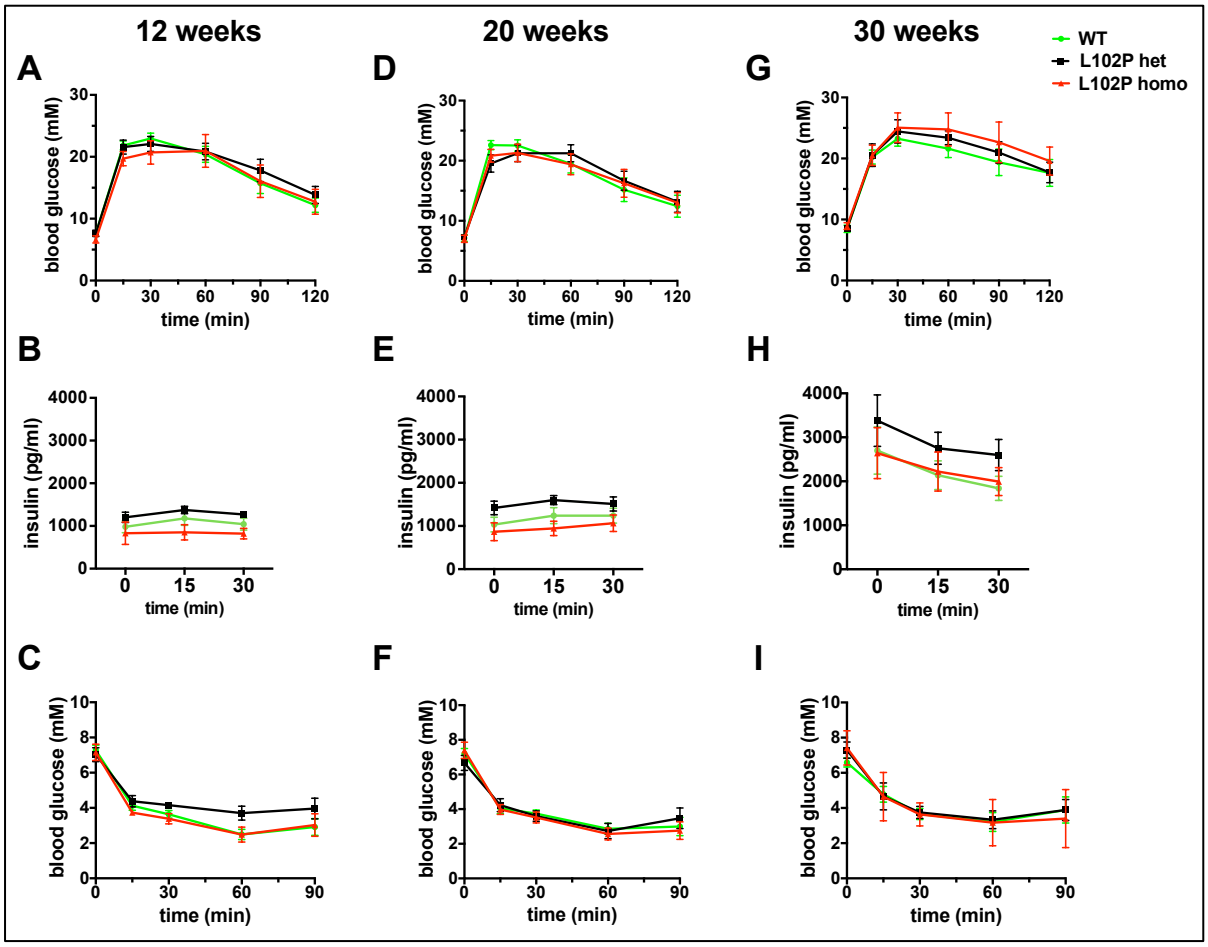

Figure 9. Homozygous L102P SIRT1 male mice exhibit no significant differences in glucose and insulin tolerance at the age of 12, 20 and 30 weeks compared to the wildtype and heterozygous mice when fed a high-fat diet. Intraperitoneal glucose tolerance tests at the age of 12 **(A)**, 20 **(D)** and 30 **(G)** weeks and the corresponding insulin measurements at time points 0, 15 and 30 **(B, E, H)**. Intraperitoneal insulin tolerance tests at the age of 12 **(C)**, 20 **(F)** and 30 **(I)** weeks. Bar graphs are expressed as mean ± SEM. Data are averages of two independent cohorts with a total of 12 (WT), 11 (het) and 7 (homo) mice. Single mice were excluded when the injection of glucose or insulin obviously did not work.

In female homozygous L102P SIRT1 mice that were fed a high-fat diet, glucose tolerance was improved at the age of 12 weeks (Fig. 10A and Fig. 11A) and impaired at the age of 20 weeks (Fig. 10D and Fig. 11B) compared to wildtype mice fed a high-fat diet.

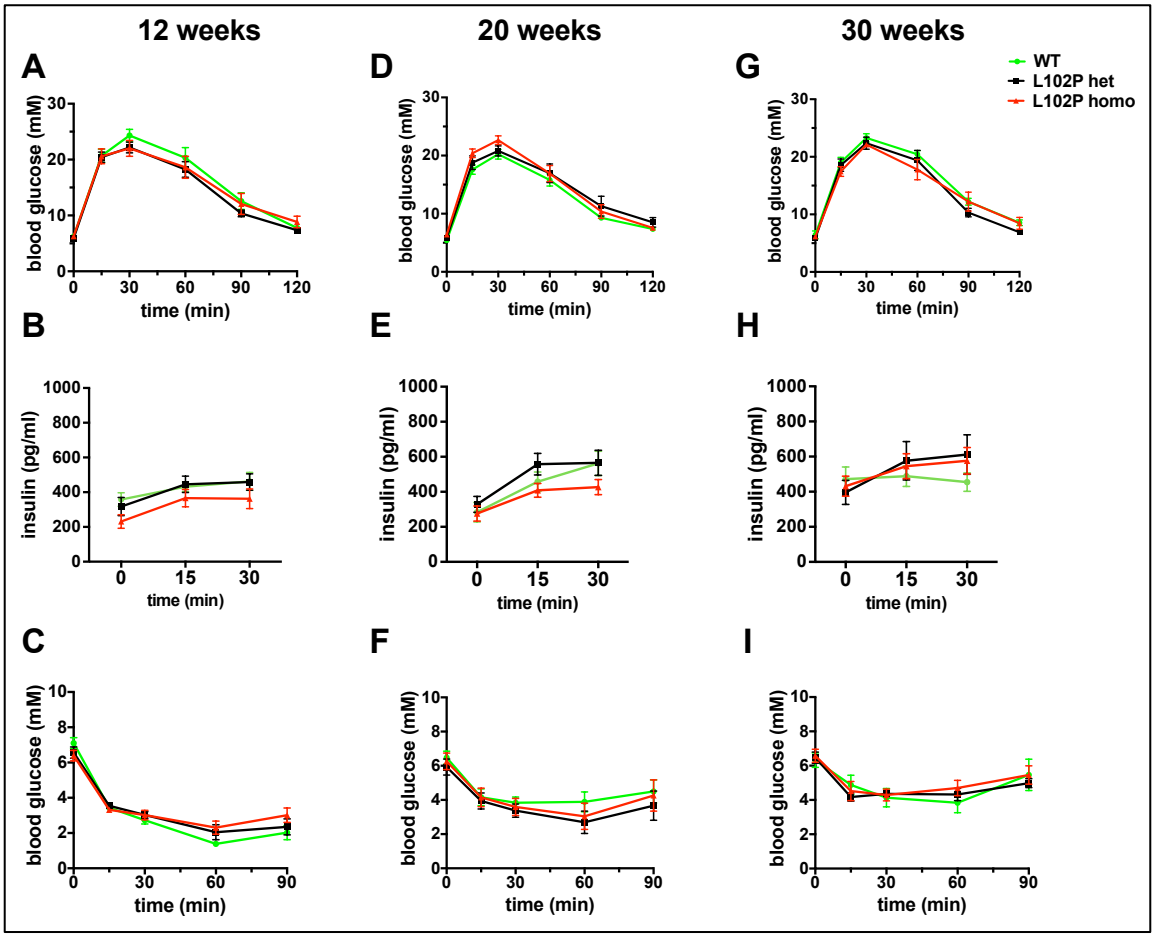

Figure 10. In homozygous L102P SIRT1 female mice at the age of 12 weeks glucose tolerance is slightly improved and at the age of 20 weeks slightly impaired compared to the wildtype mice when fed a high-fat diet. Intraperitoneal glucose tolerance tests at the age of 12 **(A)**, 20 **(D)** and 30 **(G)** weeks and the corresponding insulin measurements at time points 0, 15 and 30 **(B, E, H)**. Intraperitoneal insulin tolerance tests at the age of 12 **(C)**, 20 **(F)** and 30 **(I)** weeks. Bar graphs are expressed as mean ± SEM. Data are averages of two independent cohorts with a total of 9 (WT), 8 (het) and 10 (homo) mice. Single mice were excluded when the injection of glucose/insulin obviously did not work.

However, no significant differences were observed in glucose tolerance at the age of 30 weeks (Fig. 10G) and in insulin tolerance at all tested ages (Fig. 10C, F, I). Glucose-stimulated insulin levels did not vary throughout the whole testing (Fig. 10B, E, H).

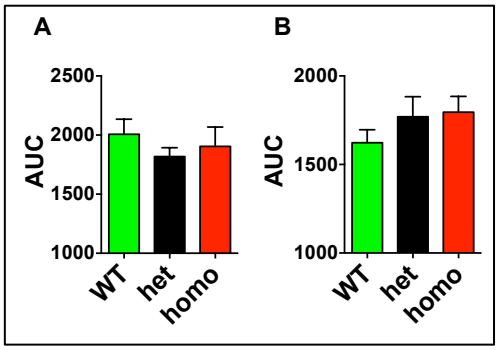

Figure 11. Glucose tolerance tests of L102P SIRT1 female mice at the age of 12 and 20 weeks fed a high-fat diet. Intraperitoneal glucose tolerance tests at the age of 12 **(A)** and 20 **(B)** weeks are expressed as area under the curve (AUC). Bar graphs are expressed as mean ± SEM.

To investigate the islet function *ex vivo*, we isolated islets of 12-week-old male mice and performed glucose-stimulated insulin secretion assays. In agreement with the improved glucose tolerance *in vivo*, islets isolated from the homozygous knock-in mice showed improved insulin secretion function compared to islets of WT and heterozygous mice (Fig. 12A). The stimulatory index, that is defined as high glucose stimulated to low glucose stimulated insulin release, was elevated in islets of heterozygous and homozygous mice compared to the control (Fig. 12B), whereas the total insulin content was the same in all three groups (Fig. 12C). However, we observed a decreased chronic insulin release in the islets of the heterozygous mice compared to the wildtype and homozygous mice (Fig. 12D).

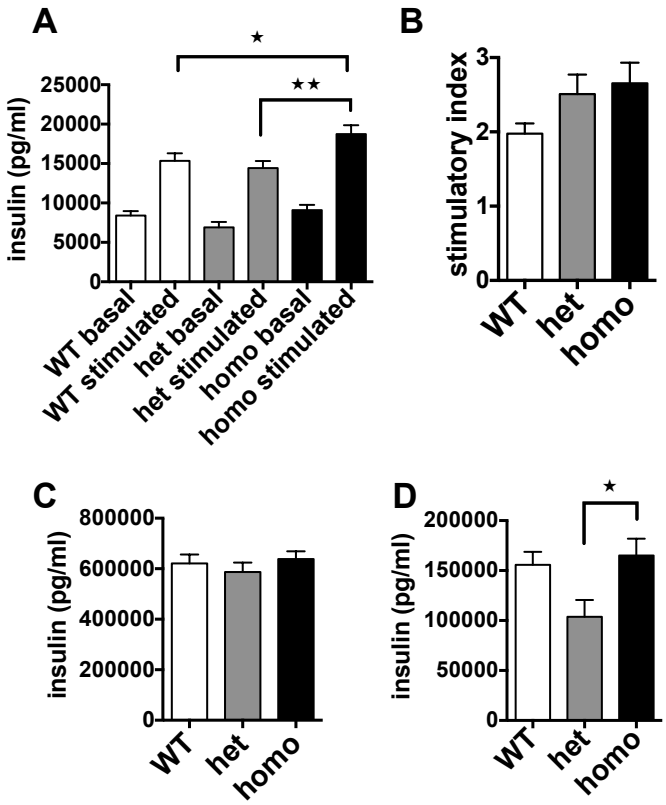

Figure 12. Glucose-stimulated insulin secretion function is enhanced in isolated islets of the homozygous L102P SIRT1 mice compared to the wildtype and heterozygous mice. (A) Glucose-stimulated insulin secretion assay in WT (white), heterozygous (grey) and homozygous mice (black). Basal = 2.8 mM glucose, stimulated = 16.7 mM glucose. **(B)** Stimulatory index of the insulin secretion was defined as high glucose stimulated to low glucose stimulated insulin release. **(C)** Total insulin content was extracted by HCl/EtOH. **(D)** Chronic insulin release was determined after 36 h of culturing. Statistics were performed using one-way ANOVA. Bar graphs are expressed as mean ± SEM. Data are averages of four (het) and six (WT and homo) independent experiments with a total of 36 (WT), 26 (het) and 38 (homo) replicates. *$p < 0.05$, **$p < 0.01$ as indicated.

4.1.4.3 Islets of homozygous L102P SIRT1 mice display elevated expression levels of inflammatory and metabolic genes

Since both transduced INS-1E and MIN6 cells were more prone to inflammation, we examined if isolated islets of L102P SIRT1 knock-in mice *ex vivo* exhibit changes in inflammatory gene expression. Therefore, we isolated islets of the three mouse strains (WT, het and homo), plated them for 36 h and treated them with or without 1 ng/ml IL-1β for 24 h prior to RNA isolation. Gene expression assays revealed a strong basal induction of islet cytokines, specifically *IL-1*β*, IL-6, Nos2* and *TNFα*, chemokines and immune cells, including CD45 (*Ptprc* gene) and *receptors for IL-22* and *IL-2*, in islets of homozygous knock-in mice compared to wildtype or heterozygous mice (Fig. 13A-H). After stimulation of the islets with IL-1β to mimic cytokine stress, all tested genes were upregulated (Fig. 13, lower part) compared to the basal levels (Fig. 13, upper part), except *Ptprc* that did not change (data not shown). The above described differences between the groups at gene expression level were even more pronounced after treatment with IL-1β, especially true for *IL-1*β, *Cxcl1*, *IL-22* and *IL-2 receptor* (Fig. 13J, M, N, O). There are no differences in gene expression levels of the macrophage chemoattractant protein-1 (*Ccl2* gene) between the groups in untreated or treated islets (data not shown).

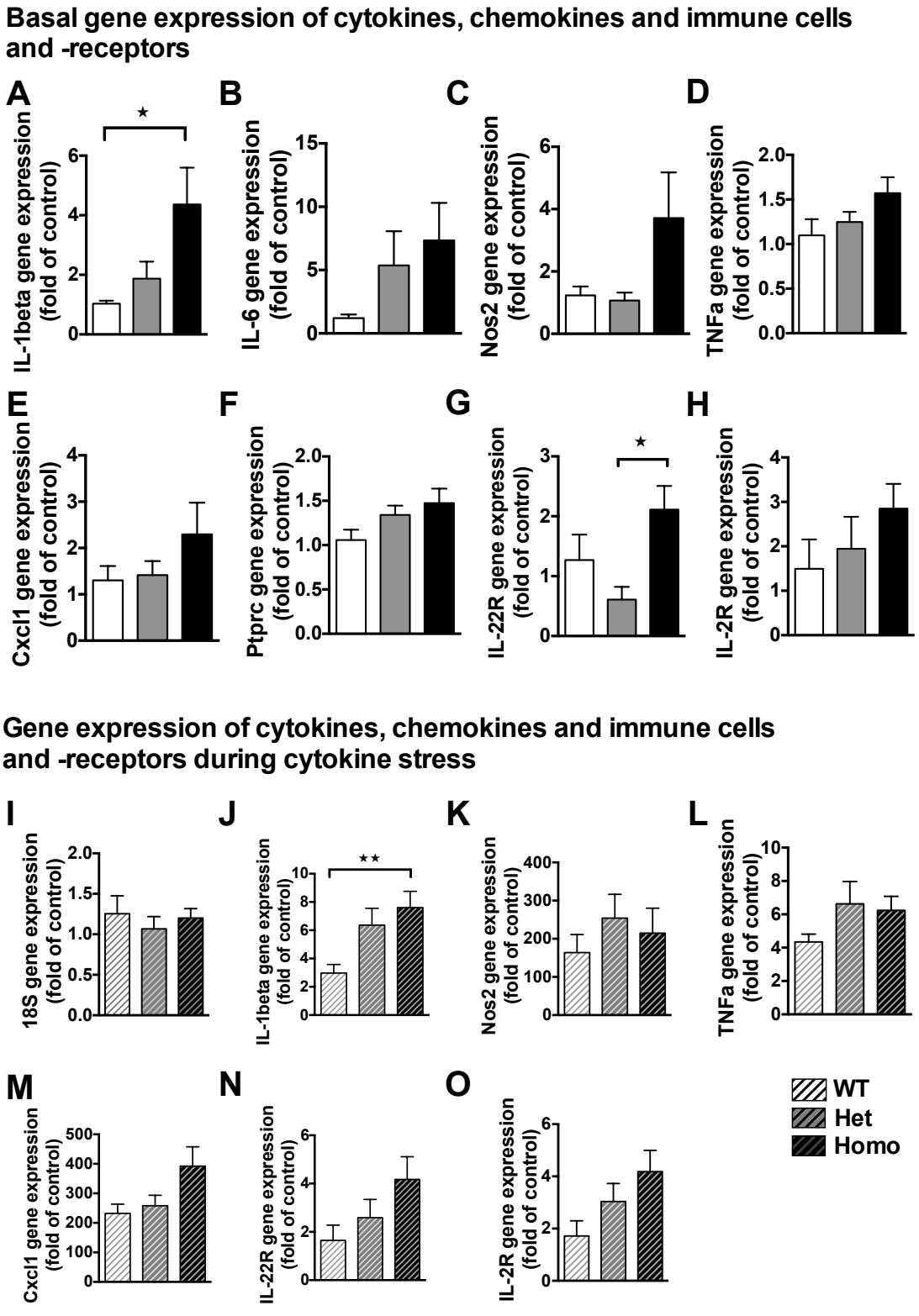

Figure 13. Islets of homozygous L102P SIRT1 mice have elevated levels of genes related to islet inflammation. Basal gene expression levels of **(A)** IL-1β, **(B)** IL-6, **(C)** Nos2, **(D)** TNFα, **(E)** Cxcl1, **(F)** Ptprc, **(G)** IL-22R, **(H)** IL-2Rα and gene expression levels of **(I)** 18S, **(J)** IL-1β, **(K)** Nos2, **(L)** TNFα, **(M)** Cxcl1, **(N)** IL-22R and **(O)** IL-2Rα during cytokine stress were determined in 80 islets of WT (white), het (grey) and homo (black) mice. Data is normalized to 18S and to the unstimulated WT control. Statistics were performed using one-way ANOVA. Bar graphs are expressed as mean ± SEM. Data are averages of three independent experiments with a total of 13 (WT), 14 (het) or 15 (homo) mice and a total of 9-12 replicates per condition. *$p < 0.05$, **$p < 0.01$ as indicated.

Several key metabolic genes, including *Ins2*, *Pdx1, glucagon* and *Foxo1*, were upregulated at mRNA level in islets of homozygous L102P mice (Fig. 14B-E). Expression levels of *Sirt1* and other members of the sirtuin family, such as *Sirt2* and *Sirt6* but not *Sirt3* (data not shown), were also elevated in homozygous mice compared to the wildtype and heterozygous mice (Fig. 14F-H). Gene expression of the uncoupling protein 2 (Ucp2), that is known to inhibit insulin secretion, was increased under basal conditions, contrasting to the higher basal insulin gene expression levels (Fig. 14I).

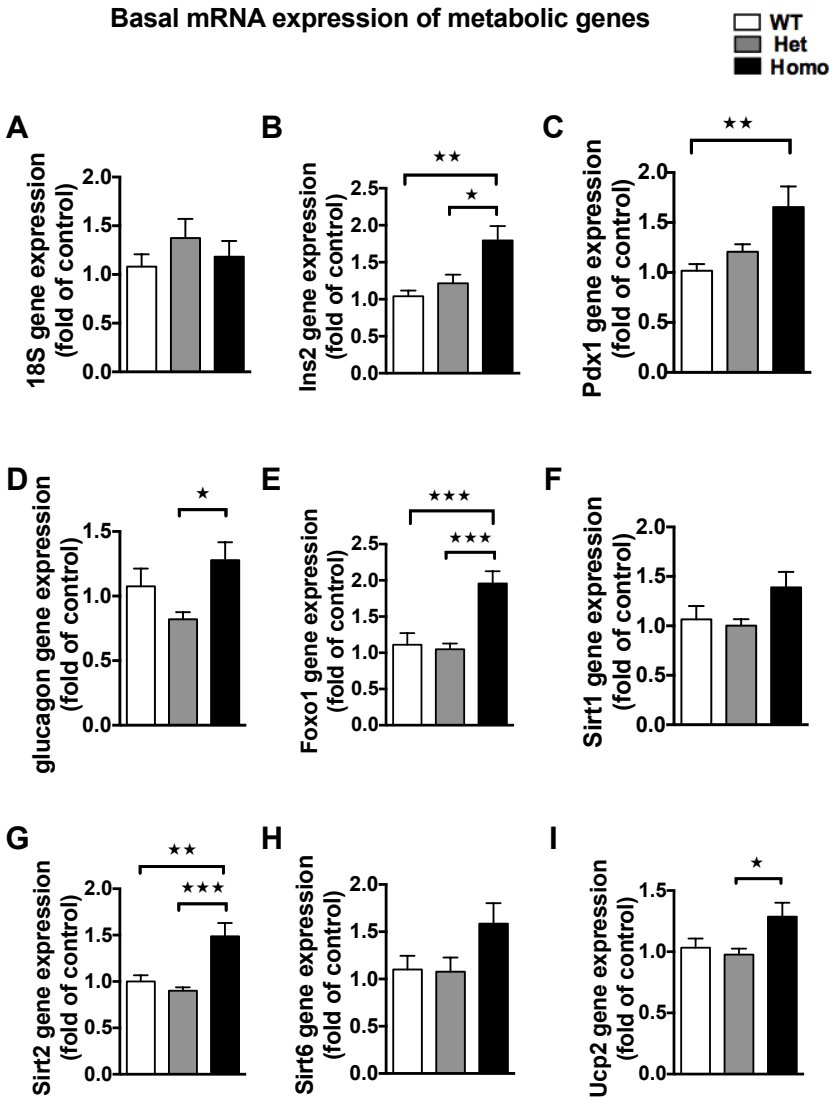

Figure 14. Islets of homozygous L102P SIRT1 mice have elevated expression levels of key metabolic genes. Basal gene expression levels of **(A)** 18S**, (B)** Ins2, **(C)** Pdx1, **(D)** glucagon, **(E)** Foxo1, **(F)** Sirt1, **(G)** Sirt2, **(H)** Sirt6 and **(I)** Ucp2 in 80 islets of WT (white), het (grey) and homo (black) mice were normalized to 18S and to the corresponding WT control. Statistics were performed using one-way ANOVA. Bar graphs are expressed as mean ± SEM. Data are averages of three independent experiments with a total of 13 (WT), 14 (het) or 15 (homo) mice and a total of 9-12 replicates per condition. *$p < 0.05$, **$p < 0.01$ as indicated.

4.1.4.4 Homozygous L102P SIRT1 mice have an autoimmune-like phenotype

Besides its role in metabolic control, SIRT1 has been demonstrated to influence immunologic processes such as T-cell tolerance and regulatory T-cell development [224]. Since the familial SIRT1 mutation causes autoimmune diseases including T1D and colitis, we investigated if there are alterations in the immune system of our mouse model. Thus, we isolated inguinal lymph node cells of all three mouse strains (WT, het and homo) and performed multiple stainings for flow cytometry to analyze the distribution of the main immune cell subsets (Fig. 15).

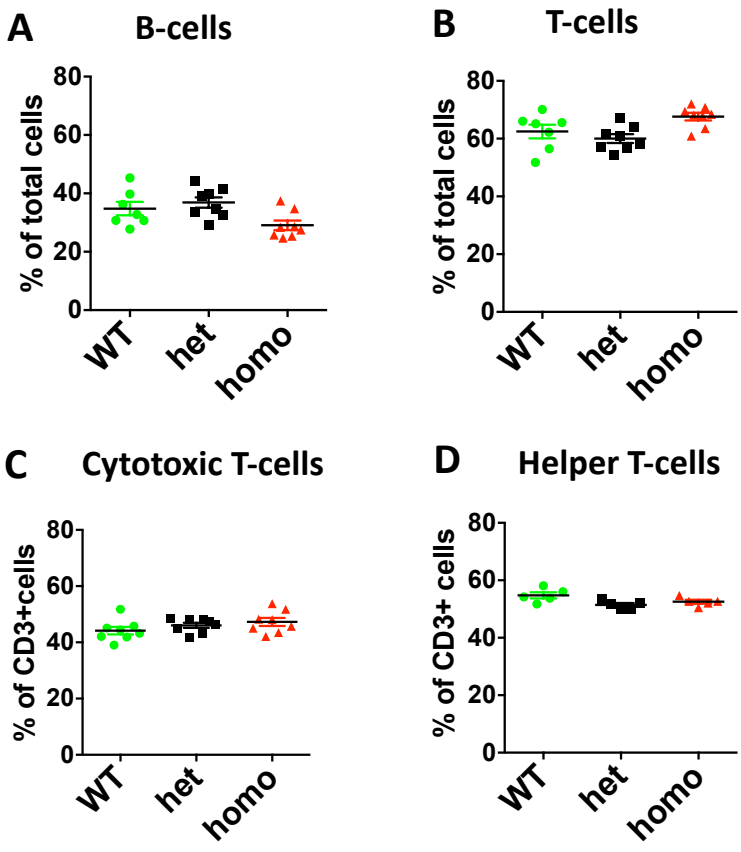

Figure 15. Immune cell subsets in inguinal lymph node cells of WT, heterozygous and homozygous SIRT1 L102P mice are comparable. Inguinal lymph node cells were isolated and stained with antibodies (eBioscience) for flow cytometry. B-cells were defined as: $CD19^+$ or $B220^+$, T-cells were defined as: $CD3^+$, cytotoxic T-cells were defined as: $CD3^+CD8^+$ and helper T-cells were defined as: $CD3^+CD4^+$. Graphs are expressed as mean ± SEM. Data are averages of three (A-C) and two (D) independent cohorts with a total of 7 (WT), 8 (het) and 8 (homo) mice.

The percentage of B-cells (defined as $CD19^+$ or $B220^+$) among all lymphocytes was slightly decreased in the homozygous mice compared to the WT and heterozygous mice (Fig. 15A), whereas the percentage of T-cells (defined as $CD3^+$) was slightly increased (Fig. 15B), without reaching statistical significance. Thus, the B- and T-cell ratio was shifted towards T-cells in the homozygous mice. No differences were observed in the proportion of cytotoxic T-cells (defined as $CD3^+CD8^+$) and helper T-cells (defined as $CD3^+CD4^+$) between the three groups (Fig. 15C+D). To further investigate the composition of the $CD4^+$ T-cells, we additionally stained for Foxp3, a transcription factor important for the function of regulatory T-cells (Tregs).

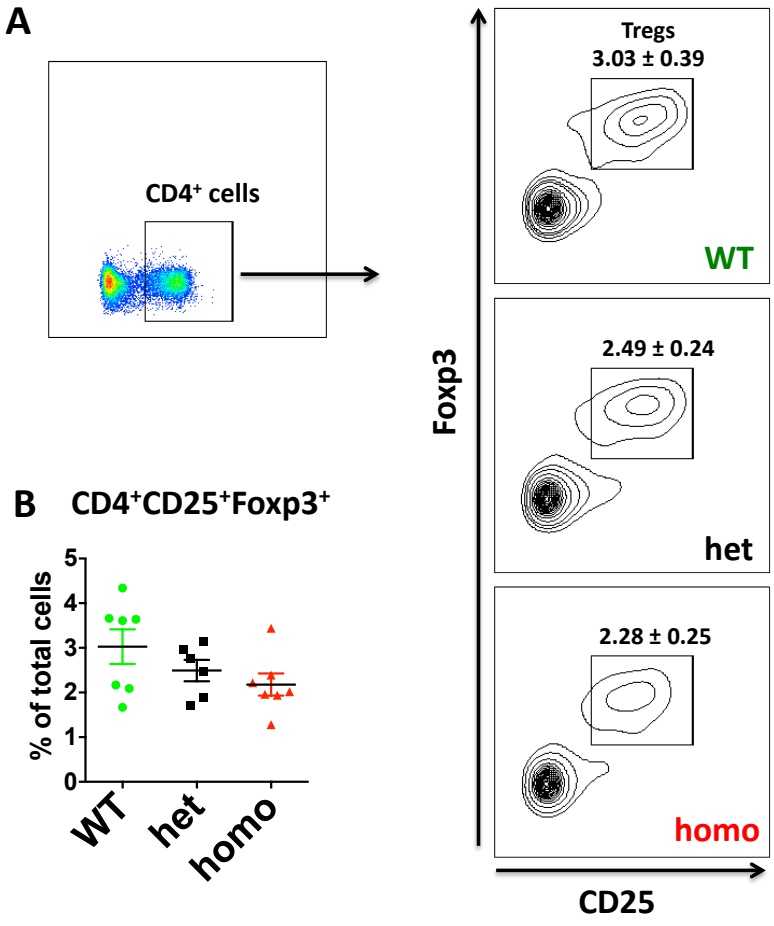

Figure 16. Homozygous L102P SIRT1 mice have lower levels of regulatory T-cells in inguinal lymph nodes compared to the wildtype mice. Inguinal lymph node cells were isolated from WT (green), het (black) and homo (red) mice and stained with antibodies (ebioscience) for flow cytometry. Regulatory T-cells (Tregs) were defined as: $CD4^+CD25^+Foxp3^+$ (one experiment was done without Foxp3 staining). **(A)** Schematic gating strategy for Tregs. **(B)** Percentage of Tregs among all lymphocytes. Bar graphs are expressed as mean ± SEM. Data are averages of three independent experiments with a total of 7 (WT), 6 (het) or 7 (homo) mice.

We detected lower levels of $CD4^+CD25^+Foxp3^+$ cells, defined as Tregs, in the lymph nodes of the homozygous knock-in mouse compared to the wildtype and heterozygous mice (Fig. 16). Since we observed reduced numbers of Tregs in the homozygous L102P SIRT1 mice pointing to a dysregulation of T-cell homeostasis, we compared the production of general immunoregulatory cytokines in the mice. Hence, we measured IL-2 concentrations in the circulation of the mice and observed significantly higher levels in the plasma of homozygous L102P mice compared to wildtype mice (Fig. 17A). Furthermore, the *ex vivo* secretion of the anti-inflammatory cytokine IL-10 by peritoneal macrophages upon stimulation with LPS, IFNγ and with or without ATP was measured and homozygous mice revealed lower IL-10 secretion levels upon stimulation compared to the wildtype mice (Fig. 17B).

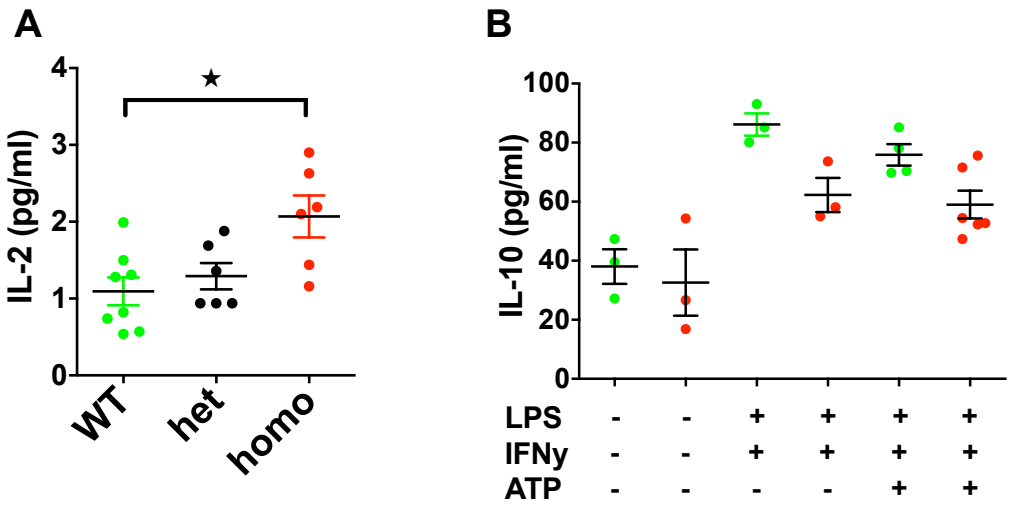

Figure 17. Homozygous L102P SIRT1 mice have elevated levels of IL-2 in the circulation and peritoneal macrophages secrete less IL-10 upon stimulation compared to wildtype mice. (A) Plasma levels of IL-2 were measured from heart blood after sacrifice of the mice (WT green, het black, homo red). **(B)** Isolated peritoneal macrophages of WT (green) and homozygous (red) mice were plated overnight and levels of IL-10 upon activation were measured in the supernatants. Statistics were performed using one-way ANOVA. Bar graphs are expressed as mean ± SEM. Data are averages of two independent cohorts with total numbers of mice: WT (9 [A] and 6 [B]), het (6 [A]) and homo (6 [A] and 4 [B]) mice. *$p < 0.05$.

4.1.5 Discussion

In this work we investigated the role of SIRT1 in the development of inflammation and autoimmune diseases including type 1 diabetes (T1D) and how a single *Sirt1* point mutation can modulate it. For our study, we used transduced rat INS-1E cells and a knock-in mouse model, which is carrying a SIRT1 point mutation that was shown to be associated with familial autoimmune diseases including diabetes and colitis [215]. We demonstrate that SIRT1 modulation by the SIRT1 point mutation causes higher susceptibility to inflammation and reduced β-cell function in the INS-1E cell model and metabolic overactivation with increased inflammation and an autoimmune-prone phenotype in the knock-in mouse model. In line with a previous study using transduced MIN6 cells ectopically expressing the mutated human *Sirt1* gene [215], we revealed reduced anti-inflammatory activities of the INS-1E β-cell line overexpressing the mutated SIRT1 compared to the wildtype SIRT1 overexpressing cell line. This was indicated by higher gene expression levels of the chemokine *Cxcl1* and enhanced production of reactive oxygen species (ROS). These results support the hypothesis that islet inflammation participates in the development of autoimmune diseases in the patients carrying the SIRT1 mutation. Furthermore, glucose-stimulated insulin secretion was impaired in the mutated INS-1E cell line, possibly caused by less protection against oxidative stress and inflammation. These findings imply that the SIRT1 mutation causes a loss of function, which is in line with various studies reporting a beneficial role of SIRT1 overexpression in the context of inflammatory processes and insulin secretion [167], [194]. Surprisingly, the overexpressing of wildtype SIRT1 in our INS-1E cell line did not protect from the induction of ROS and did not enhance glucose-stimulated insulin secretion compared to cells with endogenous SIRT1 expression (empty vector control). As described in previous studies, overexpression of SIRT1 appears to be a delicate issue, since the resulting effects are strongly dependent on the level of overexpression and on the tissue [243]. Due to the fact that SIRT1 has been shown to regulate mitophagy, specific autophagic processes in mitochondria, and mitochondrial biogenesis and function [244], we questioned if the cause of lower insulin secretion besides elevated inflammatory processes could be impaired mitochondrial function in the mutated SIRT1 overexpression cell line. No differences were detected in oxygen consumption, ATP production, mitochondrial mass and membrane potential between the cell lines.

However, mitochondrial morphology was changed in wildtype SIRT1 but not in the mutated cells during starvation. The aspect ratio, which is defined as mitochondrial length/width, was decreased in wildtype SIRT1 cells during starvation, meaning that the elongation of the mitochondria was decreased in wildtype cells but not in the mutated cells. Starvation elevates levels of cyclic adenosine monophosphate and protein kinase A leading to mitochondrial fusion and elongation and thereby determines mitochondrial fate. Longer mitochondria are protected from degradation, have more cristae and exhibit optimized ATP production during nutrient restriction [245]. However, mitophagy, which is the selective degradation of damaged mitochondria, is an important process to maintain mitochondrial function and to reduce ROS production [246]. SIRT1 has been shown to regulate the mitochondrial fusion-fission cycle by inducing mitophagy to reduce inflammation and defect mitochondria during starvation [247]. Sirt -/- mouse embryonic fibroblasts are not able to induce autophagy in response to starvation conditions [248]. Based on this knowledge, it is likely that the mutated INS-1E cells may not sufficiently activate mitophagy during starving conditions compared to the wildtype SIRT1 overexpressing cells.

Our findings suggest that the system of overexpressing human SIRT1 ectopically in INS-1E cells reflects the pathophysiological situation in the patients in the context of inflammation and β-cell dysfunction.

However, our *in vivo* data with the SIRT1 L102P knock-in mice, which is a less artificial model organism closer resembling the patients, revealed new perspectives to the SIRT1 mutation and its effects on immune responses and diabetes. In apparent contrast to the *in vitro* data with the transduced INS-1E cells, we observed enhanced glucose-stimulated insulin secretion in isolated islets of the heterozygous and homozygous knock-in mice compared to the control mice. Additionally, intraperitoneal glucose and insulin tolerance tests did not reveal differences between wildtype, heterozygous and homozygous knock-in mice, if anything, at certain ages homozygous compared to wildtype mice performed better. Given the clinical phenotype of the patients carrying the SIRT1 mutation, this finding was unexpected and led us to the hypothesis that normal function of SIRT1 is constitutively enhanced in our homozygous mouse model, possibly leading to metabolic deterioration with increasing age or additional triggers.

Further supporting our hypothesis, nearly all key metabolic genes were basally upregulated in isolated islets of homozygous mice compared to the controls. In parallel, expression levels

of numerous genes involved in islet inflammation were strongly induced, such as *IL-1*β, nitric oxide synthase (*Nos2*) and the chemokine *Cxcl1*, in line with the data of the INS-1E cells. Of note, expression of the uncoupling protein 2 (*Ucp2*) was upregulated at mRNA level, possibly compensatory to suppress the production of ROS in the inflammatory environment of the islets. Additionally, receptors for IL-2 and IL-22, which participate in the regulation of adaptive immune regulation and immune tolerance, were elevated in islets of homozygous mice at gene expression level. IL-2 is a cytokine that supports effector T-cell (Teff) differentiation but also the development of immune suppressive regulatory T-cells (Treg) [225]. IL-22 belongs to the IL-10 family of cytokines and is classically involved in Th17-induced immune responses; however, recently it was linked to the protection from obesity-induced metabolic deteriorations [28]. The upregulation of these receptors in islets could point to a compensatory mechanisms caused by the lack of IL-2 and IL-22 in homozygous mice. In line with this thinking, *ex vivo* macrophages of homozygous mice exhibited lower secretion of the anti-inflammatory cytokine IL-10 upon stimulation compared to wildtype mice in our study. Since SIRT1 is able to inhibit the production of IL-2 [218], [224] and since the lack of IL-2 has been shown to play a crucial role in the development of T1D [227], our mouse model supports the hypothesis that overactivation of SIRT1 caused by the mutation may lead to decreased IL-2 and therefore promotes autoimmunity and T1D. In contrast to our hypothesis, we observed elevated IL-2 levels in the circulation of homozygous knock-in mice compared to wildtype mice. Since IL-2 is also regulating effector T-cell development and since suppression of IL-2-induced activation of autoreactive T-cell has been used as immunotherapeutical treatment in T1D patients [226], it may well be that IL-2 has diverse functions in β-cells compared to other tissues and the circulation. In turn, IL-2 can also inhibit *Sirt1* transcription [218], possibly explaining the high IL-2 levels in the circulation as answer to overactivation of SIRT1. Due to the fact that low-dose IL-2 treatment can increase Treg recovery [231], the systemic increase of IL-2 in homozygous mice could also be a compensatory reaction to the low numbers of Tregs.

To evaluate our hypothesis of reduced immune suppressive function in the homozygous knock-in mice, we determined the immune cell subsets in lymph node cells and we indeed found less Tregs, defined as $CD4^+CD25^+Foxp3^+$ cells, in homozygous compared to wildtype mice. SIRT1 was shown to deacetylate and degrade the transcription factor Foxp3, which is constitutively expressed by Tregs [23], whereas blockage of SIRT1 improved Treg suppressive

function [232]. These studies are in line with the theory, that the mutated SIRT1 in our mouse model is overactivated and inhibits Foxp3 leading to a reduced capacity of Tregs to maintain self-tolerance. This is consistent with the results in the patients carrying the mutation; they also exhibited lower Treg levels in the blood. Additionally, preliminary data from our lab revealed reduced Treg function in homozygous SIRT1 L102P mice compared to the controls. Both the number of IL-10 producing Tregs and the functional protein cytotoxic T-lymphocyte antigen 4 (CTLA-4), that is highly expressed by Tregs and that is known to decrease the inflammatory response of antigen-presenting cells, were reduced in a pilot experiment. Furthermore, *ex vivo* activated T-cells from isolated lymph nodes of homozygous knock-in mice secreted less IL-10 than the ones from control mice. Our data are supported by recent studies; it has been shown that SIRT1 enhances the Th17/Treg ratio and the susceptibility to autoimmune diseases [233]. Additionally, inhibition of histone deacetylases (HDACs), the group of enzymes SIRT1 belongs to, was suggested to improve Treg function in autoimmune diseases including colitis [234], [235]. However, whole body and liver-specific SIRT1 knock-out mice were shown to develop autoimmune-like phenotypes [238], [249]. These discrepancies along with the divergent results of our INS-1E cell model concerning β-cell function further illustrate that SIRT1 has a variety of effects on the body, dependent on the tissue and the intensity of modulation.

However, all of this is indicative for a central and complex role of SIRT1 in T-cell biology and in the context of autoimmune diseases including T1D. Based on recent knowledge, inhibition of SIRT1 may improve the function and development of Tregs partly via IL-2 and Foxp3 enhancement and suppress the activity of Th17 effector cells. One could imagine to treat the patients carrying the SIRT1 mutation with low-dose IL-2 to increase Treg recovery, as it was tested in other autoimmune conditions [231].

We conclude that besides its function in defining metabolic changes during stress responses, SIRT1 is essentially involved in the regulation of immune reactions and inflammation. We suggest that the modification of SIRT1 through one single point mutation in a whole body knock-in mouse model causes overactivation of SIRT1 leading to enhanced insulin expression and secretion, islet inflammation and an autoimmune-like phenotype along with reduced regulatory T-cells. Thus, inhibition of SIRT1 may have therapeutic value in autoimmunity and the development of diabetes.

4.2 Manuscript No.2 - Sirtuin 1 contributes to β-cell impairment in the development of type 1 diabetes

4.2.1 Abstract

There is increasing evidence that inflammation plays a crucial effector role in the development of both type 1 and type 2 diabetes (T1D and T2D) leading to β-cell damage and β-cell death but the exact mechanisms remain to be elucidated. The NAD^+ dependent class III histone and protein deacetylase sirtuin 1 (SIRT1) has been abundantly studied in the context of longevity and metabolism but it is increasingly recognized as regulator of inflammation and the immune system. In this work, we used inducible β-cell specific SIRT1 knock-out mice to investigate how β-cell-derived SIRT1 influences glucose metabolism and the initiation and progression of diabetes. The knock-out of SIRT1 in β-cells caused impaired glucose tolerance and lower insulin levels along with defects in mitochondrial glucose sensing and increased cytokine and chemokine production in islets. However, in the context of T1D, SIRT1 in β-cells seems to be detrimental since our SIRT1 KO mice displayed a strong protection from STZ-induced diabetes and diminished loss of body weight. We propose that reduced SIRT1 activation and therefore insulin secretion during the detrimental situation of diabetes development may induce a "β-cell rest" leading to the recovery of exhausted islets along with resistance to T1D. Thus, SIRT1 expressed in β-cells is a central regulator of glucose homeostasis, islet inflammation and the development of diabetes. Based on our data, inhibition of SIRT1 in the development of T1D may have beneficial therapeutic effects.

4.2.2 Introduction

Diabetes mellitus is a multifactorial metabolic disease characterized by elevated blood glucose due to pancreatic β-cell dysfunction and insulin resistance. There is increasing evidence that inflammation plays a crucial effector role in the development of both type 1 and type 2 diabetes (T1D and T2D) leading to β-cell damage and β-cell death [33], [250]. Remaining β-cells compensate for the higher insulin demand until they fail. In T1D, additionally autoimmune components play a role and cause β-cell destruction. The mechanisms underlying the development of diabetes are not fully understood and curative treatment possibilities are still lacking.

In the last years, abundant research was done on sirtuins, a family of proteins that are involved in the regulation of metabolic homeostasis. Sirtuin 1 (SIRT1) is an NAD^+ dependent class III histone and protein deacetylase which has various tissue specific functions ranging from regulation of metabolism, circadian rhythm, cell cycle to inflammation and immune response and to general stress resistance. It is the most studied sirtuin since it has attracted a lot of interest since it was the first identified mammalian homolog of Sir2, which was shown to influence longevity in yeast [144]. SIRT1 acts as energy sensor and has been linked to the development of metabolic diseases, mainly T2D. SIRT1 overexpression was reported to improve islet function by enhancing insulin secretion [167], [150], [251] or by inhibition of inflammatory processes [194]. In many animal models of T2D, SIRT1 was shown to be a central regulator in the adaptation to diet-induced metabolic dysfunctions [172], [173]; transgenic mice lacking SIRT1 deacetylase activity are not able to acclimate to high-fat diet leading to accumulation of adipose tissue and insulin resistance [174]. It has been described that activation of SIRT1 by nicotinamide ribose (NR), a precursor of nicotinamide dinucleotide (NAD^+), can protect from HFD-induced metabolic damages through increased oxidative metabolism and mitochondrial function [175]. Nevertheless, the SIRT1 area is full of contradictory findings. SIRT1 null mice, which show developmental defects and sterility, have lower glucose and insulin levels and improved glucose tolerance [176], [150], [177]. Erion et al. showed that knockdown of hepatic SIRT1 in the context of T2D is beneficial, as indicated by decreased liver glucose production and increased whole body insulin sensitivity in a rat model [185]. SIRT1 activation in an obese mouse model of T2D reduced hyperglycemia but worsened insulin resistance and caused further body weight gain [252].

Besides its role as a metabolic modulator SIRT1 is also implicated in innate and adaptive immune response and autoimmunity via the regulation of NF-kB and AP-1 pathways and differentiation of regulatory T-cells [23]. Sirt1 -/- mice develop an autoimmune-like phenotype with increased anti-nuclear antigen antibodies [238] and recently, a point mutation in the *Sirt1* gene was linked to a familial form of T1D and autoimmune colitis [215]. In the multiple low dose streptozotocin (mldSTZ) model, a T1D model in animals, it is proposed that the SIRT1 cofactor NAD^+ is depleted by activation of PARP and DNA repair, leading to β-cell death and the induction of diabetes. In line with that, resistance to STZ-induced diabetes has been shown in PARP-1 knock-out mice [204], [205]. Several studies have proven that nicotinamide (Nam), a precursor of NAD^+ and at the same time end-product inhibitor of SIRT1, protects against STZ-induced β-cell damage and diabetes in rodents, T1D patients and in individuals with high risk [206], [207], [208], [209], [210] but it failed to prevent T1D in a large clinical trial [211]. It is still matter of debate if SIRT1 or PARP inhibition along with re-filling of the NAD^+ stocks is the predominant cause of the Nam-driven effects [212], [213]. However, an Nam derivative, isonicotinamide, which antagonizes Nam-induced SIRT1 inhibition, protects against STZ-induced hyperglycemia in high fat diet animals and β-cell damage in isolated islets [214]. Finally, whole body SIRT1 null mice but at the same time mice overexpressing SIRT1 in the β-cells are more prone to mldSTZ-induced autoimmune diabetes compared to SIRT1 +/- mice [215], [216]. Based on this contrary data, the complex role of SIRT1 in the context of diabetes is not fully understood and further investigation is needed.

In this work, we focused on the diverse functions of SIRT1 on glucose metabolism and in the development of T1D. We used inducible β-cell specific SIRT1 knock-out mice to investigate how β-cell-derived SIRT1 influences the initiation and progression of diabetes.

4.2.3 Methods

4.2.3.1 Animals

The *Pdx1creER*:*flxSirt1* mice were kindly provided by M.B. Wheeler [170] and backcrossed for five generations to obtain mice with a clean C57Bl/6N background. The background was monitored and confirmed by array testing at Biolytix. In the adult pancreas, Pdx1 promoter is expressed specifically in β-cells. According to the commonly used cre-loxP system, the injection of tamoxifen causes β-cell specific deletion of the loxP-flanked exon 4 of *Sirt1* which encodes the catalytic domain of SIRT1. Heterozygous animal breeding for the cre insertion was done to obtain flxSirt+/+_cre+/- (hereafter referred to as Cre^{+}) and flxSirt+/+_cre-/- (hereafter referred to as Cre^{-}) littermates. The knock-out of SIRT1 in β-cells was induced in 8-11 weeks old mice by daily intraperitoneal injections of 75 mg tamoxifen/kg body weight (T5648, Sigma) dissolved in corn oil or pure corn oil as control for five consecutive days. Earliest 14 days after the last injection, mice were used for experiments. In the beginning, both Cre^{+} and Cre^{-} animals were used with and without tamoxifen treatment. Since we did not see effects by the presence of cre alone or tamoxifen alone, we stopped using all the controls and only used Cre^{+} animals with corn oil ("WT") or with tamoxifen ("KO") injections. All animals were housed in a temperature-controlled room with a 12 h light/12 h dark cycle, were allowed free access to food and water and experiments were conducted according to the swiss veterinary law and to institutional guidelines.

4.2.3.2 Multiple low dose streptozotocin experiments

At the age of 10-13 weeks mice were injected intraperitoneally with 40 mg/kg body weight streptozotocin (STZ; S0130, Sigma) dissolved in 20 mM sodium citrate buffer (pH 4.5) for five consecutive days. Body weight and blood glucose levels were monitored every second day. Cages and drinking water were changed according to the need but usually twice a week. Mice were killed at day 21 after the first STZ injection or earlier if a continuation was ethically not justifiable.

4.2.3.3 Glucose tolerance tests

For intraperitoneal glucose tolerance tests, mice were fasted for 6 h in the morning and injected intraperitoneally with 2 g glucose/kg body weight. Blood glucose was measured at time points 0, 15, 30, 60, 90, and 120 min using a glucometer (Freestyle; Abbott Diabetes Care Inc., Alameda, CA) and blood was taken at 0, 15 and 30 min for measurement of plasma insulin concentrations using mouse insulin assays (Mesoscale Discovery).

4.2.3.4 Islet isolation

For mouse islet isolation, the pancreas was perfused with HBSS containing 1.4 mg/ml collagenase (Worthington, Lakewood, NJ), 10 mg/ml DNase (1:3000 to HBSS volume; Roche, Cat. No. 11284932001) and 0.5% w/v BSA (Merck, 1120180100) and digested for 30 min at 37°C and 5% CO_2, followed by washing steps and filtration through 70 and 500 µm cell strainers. Mouse islet culture medium consisted of: RPMI-1640 medium containing 11.1 mM glucose, 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamax, 50 mg/ml gentamicin, 10 mg/ml Fungizone (Gibco), and 10% fetal calf serum (FCS). Islets were cultured on extracellular-matrix-coated 24 well plates for 36 h prior to treatment with IL-1β and subsequent RNA isolation or they were used for glucose-stimulated insulin secretion experiments.

4.2.3.5 Glucose-stimulated insulin secretion

For *ex vivo* glucose-stimulated insulin secretion experiments, 25 islets/well were plated on extracellular-coated 24-well-plates for 2 days. Supernatants were stored at -20°C (chronic insulin release). Cells were incubated for 30 min in modified Krebs-Ringer bicarbonate buffer (mKRBB = 115 mM sodium chloride, 4.7 mM potassium chloride, 2.6 mM calcium chloride dihydrate, 1.2 mM mono potassium sulfate, 1.2 mM magnesium sulfate heptahydrate, 10 mM HEPES and 0.5% bovine serum albumin [pH 7.4]) containing 2.8 mM glucose. Medium was changed and islets were incubated in new mKRBB supplemented with 2.8 mM glucose for 1 h (basal insulin release), followed by 1 h in mKRBB containing 16.7 mM glucose (stimulated insulin release). Islets were extracted with 0.18 N hydrogen chloride in 70% ethanol for 2 h in room temperature to determine insulin content. Insulin concentrations

were measured using mouse/rat insulin kits (Mesoscale Discovery, Rockville, MD). Stimulatory index was determined as ratio of stimulated to basal insulin release.

4.2.3.6 RNA isolation and quantitative PCR

After isolation, 80 islets were plated on extracellular matrix-coated 24-well-plates for 36 h. Medium was changed and islets were treated with or without 1 ng/ml recombinant mouse IL-1β (R&D Systems, 401-ML) for 24 h. Total RNA was extracted using the Nucleo Spin RNA II kit (Macherey-Nagel, Düren, Germany). The isolated RNA was reverse transcribed using Superscript II (Invitrogen) and random hexamer primers (Microsynth, Balgach, Switzerland). Quantitative PCR was done with the real-time PCR system 7500 (Applied Biosystems) and the following mouse TaqMan assays (Life Technologies) were used: β-actin: Mm00607939_s1, Sirt1: Mm01168519_m1, Sirt2: Mm01149204_m1, Sirt3: Mm00452131_m1, IL-6: Mm00446190_m1, IL-1β: Mm00434228_m1, Cxcl1: Mm00433859_m1, Ins2: Mm00731595_gH, Gcg: Mm00801714_m1, UCP2: Mm00627599_m1, Emr1: Mm00802529_m1, Ptprc: Mm01293577_m1 and Ccl2: Mm00441242_m1.

4.2.3.7 Oxygen consumption assay

Seahorse Bioscience technology was used to test mitochondrial function of isolated islets. Pancreatic mouse islets were isolated and incubated in mouse islet medium (see chapter "Islet isolation") over night at 37°C. The next day, islets were handpicked and 70 islets/well were seeded in a XF24 islet capture microplate (Seahorse Bioscience) in unbuffered RPMI-1640 medium (R1383, SIGMA) supplemented with 3 mM glucose and 0.5% BSA and were incubated at 37°C without CO_2 for 2 h. To test the reaction to high glucose conditions, ATP turnover, maximal respiratory capacity and non-mitochondrial respiration of the islets, 20 mM glucose, 5 µM oligomycin, 3 µM of the uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and 5 µM rotenone were injected successively and oxygen consumption rates and extracellular acidification rates were measured.

4.2.4 Results

4.2.4.1 Deletion of SIRT1 in β-cells does not influence the vitality of the mice

The knock-out of SIRT1 in β-cells was induced via daily intraperitoneal tamoxifen injections for five days in Cre^+ mice. The inducible deletion strategy is illustrated in Figure 1A. Control mice (Cre^+) were injected with corn oil. Earliest 14 days after the last injection mice were used for *in vivo* experiments or sacrificed for *in vitro* tests. The knock-down of SIRT1 in islets was confirmed at the gene expression level by quantitative PCR (Fig. 1B left) and at the protein level by western blotting using anti-mouse Sir2 antibodies (corresponding murine SIRT1; Fig. 1C).

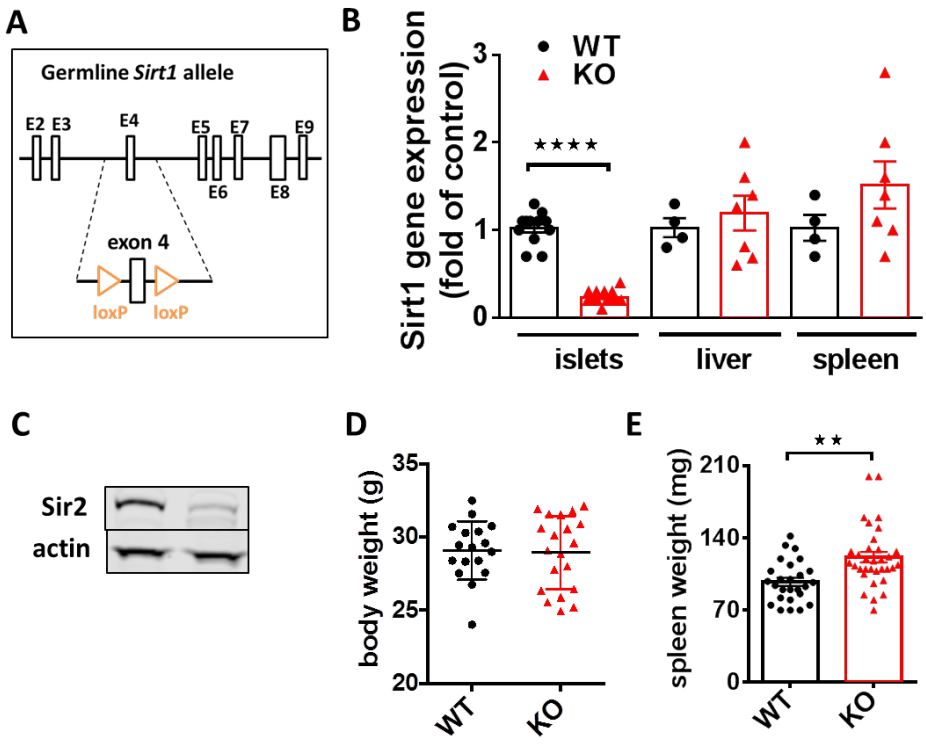

Figure 1. SIRT1 specifically knocked out in β-cells does not influence vital functions of the mice. (A) Schematic *Sirt1* exon 4 locus. Adapted from [170]. **(B)** Confirmation of specific knock-out of SIRT1 in islets but not in liver and spleen by qPCR. Gene expression levels are normalized to actin and to the WT control of each tissue**. (C)** Absence of SIRT1 protein in islets of the knock-out mice, visualized by a representative western blot picture using anti-mouse Sir2 antibodies (Millipore) and the actin control. **(D)** Body weight and **(E)** spleen weight two weeks after the first tamoxifen injection. Bar graphs are expressed as mean ± SEM. Data are averages of three (B, liver & spleen) or five (B, islets) independent experiments. In (D) and (E) one dot represents one mouse. Statistics were performed using student *t* test. **p < 0.01, ****p < 0.0001.

There is still residual SIRT1 gene and protein detection in the islets probably due to expression in α-cells and other non β-cells. *Sirt1* gene expression levels in other tissues such as liver and spleen were not changed in the SIRT1 KO mice compared to the controls (Fig. 1B middle and right). Compensatory upregulation of genes of other sirtuin family members, such as *Sirt2* or *Sirt3*, was not observed in the islets of SIRT1 KO mice (data not shown). No obvious change of behavior, health and body weight of the mice was noted due to the knock-out of SIRT1 (Fig. 1D). However, we observed splenomegaly in the mice with the SIRT1 deletion (Fig. 1E) but not in Cre^- mice with or without tamoxifen treatment (data not shown), possibly pointing to systemic inflammatory processes caused by the β-cell specific knock-out of SIRT1.

4.2.4.2 SIRT1 knock-out in β-cells deteriorates glucose tolerance, changes metabolic key gene expression and increases islet inflammation

It has been shown that SIRT1 in β-cells is an important regulator of metabolic homeostasis [170]. To confirm these data obtained with mice on a mixed genetic background in our backcrossed mice, we performed intraperitoneal glucose tolerance tests in Cre^+ and Cre^- mice with oil or tamoxifen injections. As shown in Figure 2A+B, the β-cell specific SIRT1 knock-out mice ("SIRT1 KO") had a significantly impaired glucose tolerance and lower insulin secretion when compared to the control mice treated with oil ("WT"). Cre or tamoxifen itself did not influence metabolic parameters since Cre^- mice displayed no differences in glucose tolerance or insulin secretion when injected either with oil or with tamoxifen (Fig. 2C+D). Therefore, we did not include Cre^- controls in the following experiments.

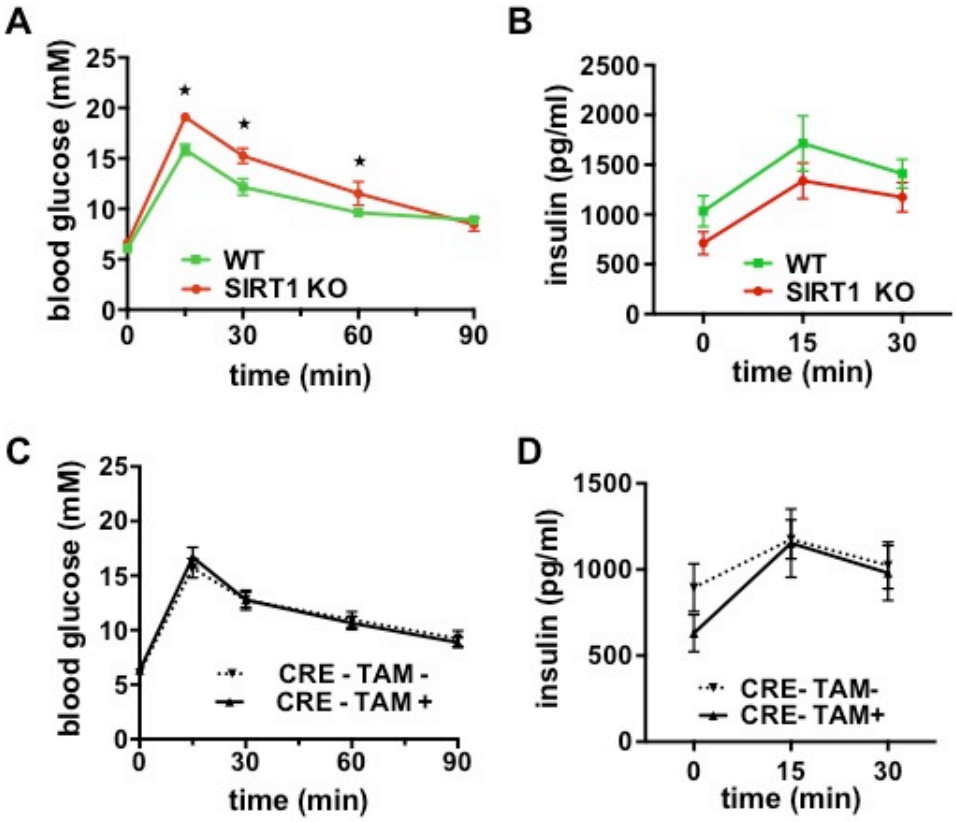

Figure 2. **SIRT1 knock-out in β-cells deteriorates glucose tolerance and insulin secretion.** Intraperitoneal glucose tolerance tests in Cre^+ **(A)** and Cre^- **(C)** mice with injections of tamoxifen (A in red, C solid line) and oil (A in green, C dashed line) and corresponding blood insulin measurements at time points 0, 15 and 30 min **(B) + (D)**. Statistics were performed using the student *t* test at the indicated time points. Bar graphs are expressed as mean ± SEM. Data are averages of three independent experiments with a total of 7-10 mice per group.*$p < 0.05$.

In line with the decreased basal plasma insulin *in vivo*, we saw lower gene expression levels of *Ins2,* encoding insulin, in *ex vivo* isolated islets from the SIRT1 KO mice compared to the controls (Fig. 3A). Additionally, relative gene expression levels of *glucagon* were increased and levels of *Pdx1*, an insulin gene specific transcription factor, decreased (Fig. 3B+C), whereas there was no difference in mRNA levels of the uncoupling protein *Ucp2* (Fig. 3D) that is regulating insulin secretion.

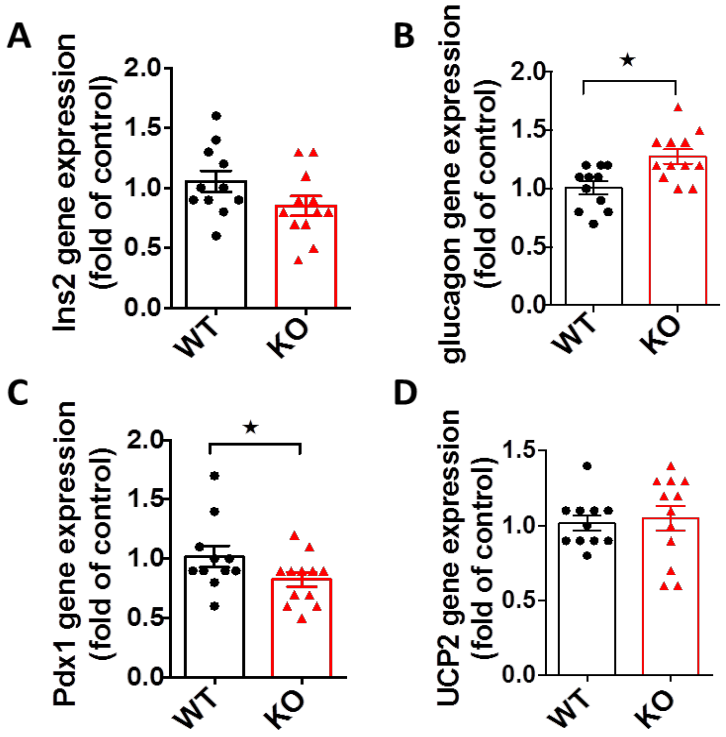

Figure 3. **SIRT1 knock-out in β-cells influences the expression of key metabolic genes *ex vivo*. (A)** *Ins2*, **(B)** *glucagon*, **(C)** *Pdx1* and **(D)** *Ucp2* gene expression in islets of mice lacking SIRT1 in β-cells (KO in red) versus controls (WT in black). Relative expression levels were normalized to actin. Statistics were performed using the student *t* test. Bar graphs are expressed as mean ± SEM. Data are averages of three independent experiments. *$p < 0.05$.

However, *ex vivo* functional experiments with isolated islets of the knock-out mice were not consistent with the *in vivo* findings. Glucose-stimulated insulin secretion assays revealed no significant differences between the groups (Fig. 4A). Unexpectedly, the stimulatory index was slightly higher in the SIRT1 KO islets (Fig. 4B), whereas total insulin content was the same (Fig. 4C). We observed a mild decrease in chronic insulin release in the SIRT1 KO group compared to the WT control (Fig. 4D).

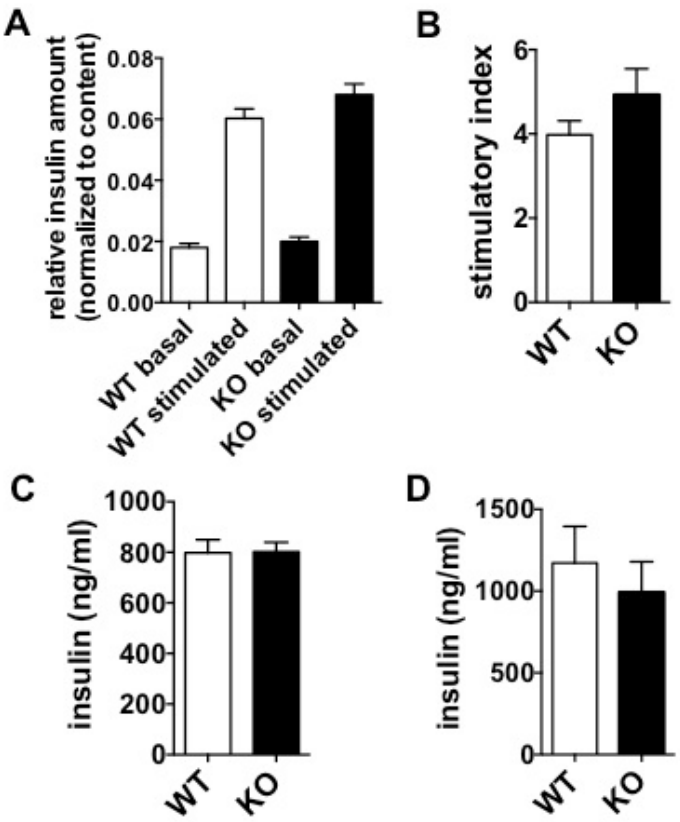

Figure 4. **SIRT1 knock-out in β-cells does not change glucose-stimulated insulin secretion *ex vivo*. (A)** Glucose-stimulated insulin secretion assay in WT (white) and SIRT1 KO mice (black), relative insulin amount is normalized to total insulin content. Basal = 2.8 mM glucose, stimulated = 16.7 mM glucose. **(B)** Stimulatory index of the insulin secretion was defined as stimulated to basal insulin release. **(C)** Total insulin content was extracted by HCl/EtOH. **(D)** Chronic insulin release after 36 h culturing. Statistics were performed using the student *t* test. Bar graphs are expressed as mean ± SEM. Data are averages of six independent experiments.

To further understand these findings, we tested the mitochondrial function of the islets with the Seahorse technology (Bucher AG). Starting with a higher basal oxygen consumption rate (OCR), which reflects mitochondrial respiration, under low glucose conditions, the islets of the SIRT1 KO mice compared to the WT control mice reached a slightly lower OCR level after injection of 17 mM glucose (Fig. 5A). We did not observe differences after treatment with oligomycin, FCCP and rotenone indicative for ATP turnover, maximal respiration rate and non-mitochondrial respiration, respectively (Fig. 5A). Extracellular acidification rates (ECAR), representing glycolytic processes, were slightly increased in islets of the KO mice upon high glucose stimulation without reaching significance (Fig. 5B).

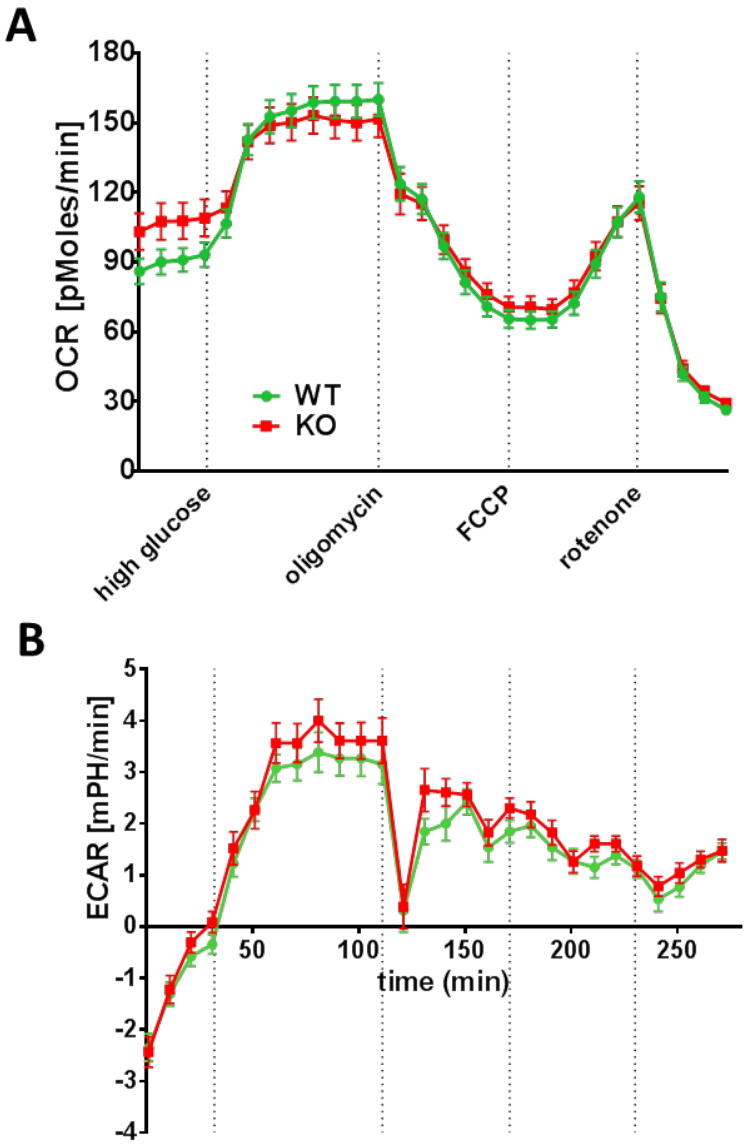

Figure 5. Mitochondrial glucose sensing is impaired in islets of mice lacking SIRT1 in β-cells. (A) Oxygen consumption rates (OCR) and **(B)** extracellular acidification rates (ECAR) of isolated WT and SIRT1 KO islets determined by the Seahorse instrument. Successive injections of 17 mM glucose (end concentration 20 mM), the ATP synthase inhibitor oligomycin, the uncoupler FCCP (carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone) and rotenone were done on 70 islets/well in unbuffered environment. Data are averages of three independent experiments. Bar graphs are expressed as mean ± SEM.

Since SIRT1 was shown to protect β-cells from the induction of pro-inflammatory cytokines in islets [192], [194] we wondered if there is a link between deterioration of glucose tolerance and islet inflammation in our SIRT1 KO mice. Therefore, we isolated islets of SIRT1 KO mice and controls and treated them with or without 1 ng/ml IL-1β for 24 h *ex vivo*. The induction of pro-inflammatory cytokines and chemokines, such as *IL-1*β, *IL-6* and *Cxcl1* were elevated in mice lacking SIRT1 in β-cells after stimulation with IL-1β (Fig. 6A-C). Basally,

mRNA levels of the macrophage marker *Emr1,* which is encoding F4/80, were increased in the islets of the SIRT1 KO mice and after stimulation *Ptprc,* which is encoding the general immune cell marker CD45 (Fig. 6D+E). Interestingly, the gene expression of *Ccl2*, a monocyte chemotactic protein, in islets of the SIRT1 KO mice was significantly lower (Fig. 6F).

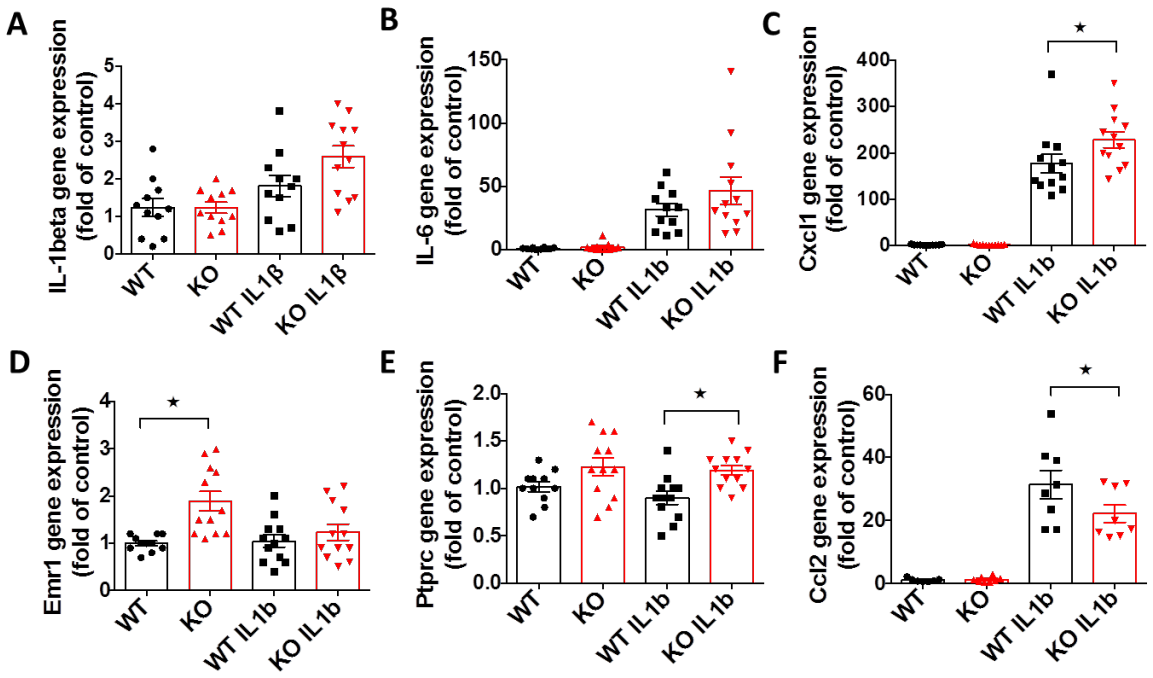

Figure 6. The induction of pro-inflammatory cytokines, chemokines and immune cell markers is increased in islets of mice lacking SIRT1 in β-cells. Relative gene expression levels of **(A)** *IL-1*β, **(B)** *IL-6*, **(C)** *Cxcl1*, **(D)** *Emr1* encoding F4/80, **(E)** *Ptprc* which is encoding CD45 and **(F)** *Ccl2* encoding MCP-1 in islets of mice lacking SIRT1 in β-cells (red) and control mice (black) with and without treatment with 1 ng/ml IL-1β for 24 h. Data are averages of three independent experiments. Bar graphs are expressed as mean ± SEM. Statistics were done using ANOVA. *$p < 0.05$.

4.2.4.3 SIRT1 knock-out in β-cells protects mice from multiple low dose streptozotocin-induced hyperglycemia

Since islet inflammation is also associated with T1D and the lack of SIRT1 in mice was shown to worsen [215] but also to decrease the susceptibility to T1D [216], we wanted to test the influence of β-cell specific SIRT1 knock-down on the onset of T1D. Therefore we used the multiple low dose streptozotocin model (mldSTZ), which is characterized by destruction of β-cells and islet immune cell infiltrations. Two weeks after the induction of the SIRT1 knock-out, mice were injected with STZ for five consecutive days and blood glucose was monitored

the following 21 days. We observed a strong protection from the STZ-induced hyperglycemia (defined as blood glucose >10 mM) in the SIRT1 KO group compared to the WT mice (Fig. 7A).

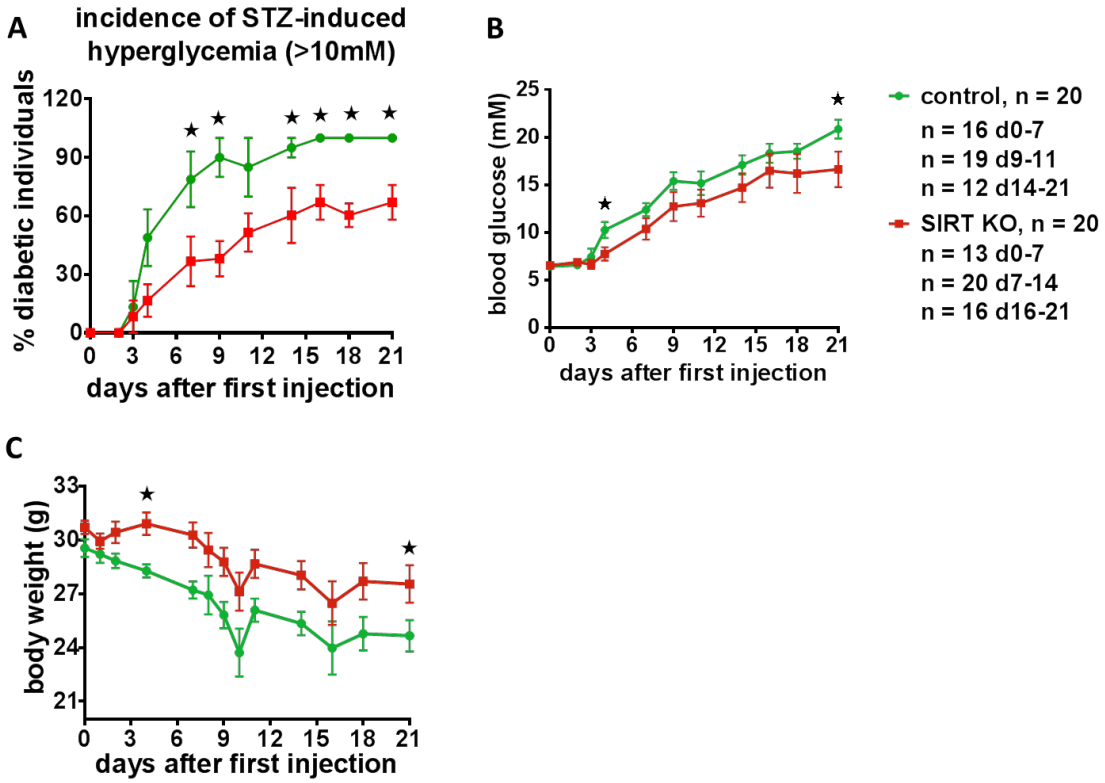

Figure 7. Knock-out of SIRT1 in β-cells protects mice from STZ-induced hyperglycemia. (A) Incidence of STZ-induced hyperglycemia, defined as blood glucose >10 mM. SIRT1 KO mice are shown in red and control mice in green. **(B)** Random blood glucose was taken in the afternoon during the STZ experiment. **(C)** Body weight of the SIRT1 KO (red) and control mice (green). Bar graphs are expressed as mean ± SEM. Data are averages of four independent experiments with a total of 12-20 mice dependent on the day, as described in the graph (d=day). Statistics were done using the student *t* test for the specific time points. *p < 0.05.

The difference in plasma glucose was already evident at day 4 after the first STZ injection and was present until the end of the experiment. Contrasting to the WT mice, where 100% of the animals became diabetic, in the KO group only 67% of the mice became diabetic. The absolute values of random blood glucose measurements of the KO mice always stayed below the values of the control group (Fig. 7B). Mice of both groups were losing body weight during the experiment, which is associated with the severity of the diabetes. The lower number of diabetic mice was also reflected by less body weight reduction in the SIRT1 KO mice compared to the controls (Fig. 7C).

4.2.5 Discussion

In this study we demonstrate that SIRT1 expressed in β-cells plays a crucial role in glucose homeostasis, islet inflammation and the development of diabetes. The knock-out of SIRT1 in β-cells caused impaired glucose tolerance and lower insulin levels along with defects in mitochondrial glucose sensing and increased cytokine and chemokine production in islets. However, in the context of type 1 diabetes, SIRT1 activity in β-cells seems to be detrimental since our SIRT1 KO mice were protected from STZ-induced diabetes and the loss of body weight.

In line with former studies of Luu et al. [170], SIRT1 deletion in β-cells leads to impaired glucose tolerance and insulin secretion in our mouse model. However, the differences between SIRT1 KO and WT mice were less pronounced than those reported by Luu et al. This could be explained by different methods that have been used: In our experiments, mice were fasted for 6 h and injected intraperitoneally with glucose, whereas Luu et al. fasted for 14 h and performed oral glucose tolerance tests, which involve incretin release and thereby boost insulin secretion. Furthermore, we have used littermate and fully backcrossed mice, while to our knowledge Luu et al. used mice on a mixed background.

Ex vivo after islet isolation, we did not see differences in glucose-stimulated insulin secretion experiments. If anything, the stimulatory index of the SIRT1 KO islets was increased compared to the WT control mice. This finding contrasts previous studies showing that islets of β-cell specific SIRT1 knock-out mice and β-cells treated with SIRT1 siRNA exhibit blunted insulin release upon glucose stimulation [170], [150]. It has been discussed earlier that pronounced *in vivo* effects can change in *ex vivo* experiments [253], [254]; what likely may be the case in our glucose-stimulated insulin secretion experiments. Certain deteriorating factors may be missing after islet isolation and plating on extracellular-matrix-coated plates for 36 h may lead to islet recovery and improved function, covering negative effects observed *in vivo*.

Furthermore, the basal expression of key metabolic genes including *insulin*, *glucagon* and *Pdx1* was altered in isolated islets of the SIRT1 KO compared to the control mice. Lower *insulin* and *Pdx1* mRNA levels *ex vivo* are in agreement with lower insulin levels and the deterioration of glucose tolerance *in vivo.* Accordingly, SIRT1 has been reported to promote insulin and *Pdx1* expression [193], [255].

Previous studies have shown that the expression of Ucp2, which decreases insulin secretion, is upregulated in SIRT1 knock-out models and downregulated in SIRT1 overexpressing models [167], [150]. In contrast to that, *Ucp2* gene expression was unchanged in isolated islets of our mice, which is congruent with the study from Luu et al. [170] and points to an Ucp2 independent mechanism in the deterioration of insulin secretion. The determination of Ucp2 protein levels would be helpful to further evaluate this finding.

However, in our study we revealed defects in mitochondrial function of the SIRT1 KO islets. There was a pronounced higher basal oxygen consumption rate and a decreased coupling efficiency, indicating the translation of high glucose levels to elevated oxygen consumption. In former studies, SIRT1 has been shown to regulate mitochondrial biogenesis and function in metabolically active tissues [256] including β-cells [170]. Since basal levels of insulin were lower in islets and in the circulation of the SIRT1 KO mice, the observed higher basal oxygen consumption rates could be due to compensatory effects. These higher basal levels of OCR in the islets of the SIRT1 KO mice may blunt the reaction to the high glucose stimulation. In parallel, glycolytic processes were slightly increased, as indicated by elevated extracellular acidification rates. Since there were no differences in non-mitochondrial and maximal respiration, severe mitochondrial failure can be excluded.

There are several explanations why changed oxygen consumption rates are not reflected by impaired glucose-stimulated insulin secretion in isolated islets of SIRT1 KO mice. As described above, our *ex vivo* insulin secretion experiments may be misleading. Direct comparison of glucose-stimulated insulin secretion and oxygen consumption is biased by experimental differences since the secretion assays are performed 36 h after islet isolation and the mitochondrial testing 12 h afterwards. Possibly, the islets used for the glucose-stimulated insulin secretion experiments had more time to recover from the isolation stress and may have improved their insulin secretion capacity. Additionally, islets used for glucose-stimulated insulin secretion experiments were plated on extracellular matrix-coated plates where they are less disturbed by hypoxia in the core of the islets, compared to the islets cultured in suspension for functional mitochondrial testing.

Islet inflammation has been shown to be a main contributor to the development of glucose intolerance and diabetes [257]. Previous studies have linked the absence of SIRT1 to cytokine-induced inflammation in various tissues including adipocytes, myeloid cells and β-cells [196], [199], [258], [194]. We also observed increased gene expression of pro-

inflammatory cytokines and chemokines such as *IL-1*β, *IL-6* and *Cxcl1* in the islets of SIRT1 knock-out mice upon stimulation with IL-1β. Additionally, immune cell and macrophage markers including *Ptpcr* and *Emr1* were upregulated in SIRT1 KO islets, indicative for elevated islet infiltration and inflammation, whereas the expression for the macrophage chemoattractant *Ccl2* was significantly lower. We do not have an explanation for the lower *Ccl2* levels but it may be due to compensatory effects to inhibit additional macrophage infiltration. However, these findings further support the importance and the distinct functions of β-cell SIRT1 in immunity and diabetes.

Finally, our *in vivo* data of the mldSTZ experiments led to a new way of thinking about the role of SIRT1 in the onset of T1D. The mice lacking SIRT1 in β-cells were protected from mldSTZ-induced hyperglycemia; in contrast to the wildtype mice they did not reach 100% diabetes induction and dropped less body weight throughout the experiment. The literature on the role of SIRT1 in the development of diabetes is contradictory and full of open questions. Nonetheless, it has been shown in whole body knock-out mice that lack of SIRT1 is increasing the susceptibility to STZ-induced hyperglycemia [215]. The comparison of these global SIRT1 knock-out mice with our findings in the β-cell specific SIRT1 knock-out mice conveys a subordinate role of β-cell-derived SIRT1 in the manifestation of the demonstrated STZ-induced deteriorations. In absolute agreement with our data, it was shown that mice overexpressing SIRT1 in β-cells are more prone to STZ-induced diabetes [216].

During the demanding process of diabetes induction, remaining islets have a high workload but try to continue their hormone production. We hypothesize that this exhaustion may be improved by the induction of a "β-cell rest" via knock-out of SIRT1 in β-cells, promoting longer islet survival and function along with resistance to T1D. The positive effects of β-cell rest have been shown *in vitro* and in clinical studies [259], [260]. It is suggested that β-cells with increased workload may recover when insulin secretion is inhibited or exogenous insulin is offered.

One should take into consideration that mldSTZ experiments in the context of SIRT1 are delicate since both substances are known to consume NAD^+, either by STZ-induced activation of PARP or because it is used as cofactor for SIRT1 [204], [136]. It is possible that the beneficial effects of the SIRT1 KO in β-cells on STZ-induced hyperglycemia arose from increased availability of NAD^+ due to the lack of SIRT1. Based on occurring death cases induced by the SIRT1 activator SRT1720 in a mouse model of T1D [166] and on unpublished

experiments of our group with NOD mice, which are independent of NAD^+ depletion by PARP, it is more likely that SIRT1 activation itself is detrimental in the context of T1D.

It has been suggested in numerous studies that overexpression and overactivation of SIRT1 in different tissues and situations does not necessarily have beneficial effects. It was shown that strong (12.5-fold) heart-specific overexpression of SIRT1 leads to the induction of oxidative stress and cardiac dysfunction [243]. Furthermore, SIRT1 was linked to the attenuation of β-cell expansion [261], to decreased insulin sensitivity [185] and to worsening of insulin resistance in diabetic mice [252]. Overexpression of SIRT1 failed to protect mice from HFD-induced obesity [169], [172] and impairment of insulin sensitivity [182]. Additionally, SIRT1 overexpression in rheumatoid arthritis is suggested to contribute to pro-inflammatory cytokine production and apoptosis resistance [262]. As mentioned above, the synthetic small SIRT1 activator SRT1720 that was shown to be beneficial for metabolic and mitochondrial function in animal models of T2D [164], [160], [263], [165] failed to improve metabolic conditions in other mouse studies and caused accumulated death cases, shown by Pacholec et al. [166] and by our research group in NOD mice (unpublished data). These findings further suggest a negative role of SIRT1 overactivation in the context of diabetes.

In the last few years, it has been pointed out that inhibition of histone deacetylases, the group of enzymes where SIRT1 belongs to, can improve metabolic functions, β-cell inflammation and insulin sensitivity via enhanced activity of PPARγ or glucose uptake in muscle [264], [200]. Additionally, the inhibition of lysine deacetylases in the NOD mouse model of T1D resulted in decreased pro-inflammatory factors in β-cells and leukocytes [265]. SIRT1 inhibition as possible treatment has been tested in other disease areas as well. Huntington`s disease (HD) is a neurodegenerative disease known to develop through transcriptional dysregulation and dysfunctional protein acetylation. The inhibition of deacetylation by SIRT1 blockage was well tolerated in a human study [266] and resulted in improvement of HD pathology in several cell and mouse models [267]. Thus, inhibition of SIRT1 as histone and protein deacetylase may also have beneficial effects on the development of T1D.

To summarize, we propose that SIRT1 expressed in β-cells is a central regulator of glucose homeostasis, islet inflammation and the development of diabetes. It has diverse effects: on one hand, deletion of SIRT1 in β-cells causes impaired glucose tolerance, mitochondrial defects and elevation of inflammatory gene expression in islets. On the other hand, in the context of T1D, knock-out of SIRT1 in β-cells prevents the induction of hyperglycemia and loss of body weight.

5 General Discussion and Conclusion

5.1 Angiotensin II

In the included publication (chapter 3.1) we present the negative influence of Ang II on glucose metabolism, independently of vasoconstriction and insulin sensitivity. In high-fat diet mice as a model of type 2 diabetes, Ang II treatment results in β-cell dysfunction and impaired insulin secretion along with islet inflammation. We uncover that Ang II is an inflammatory modulator involved in NF-κB and IL-1β-mediated islet inflammation *in vitro* and *in vivo*. Furthermore, we demonstrate β-cell specific pro-apoptotic and mitochondria-damaging effects of Ang II.

The discrepancy between our *in vivo* and *ex vivo* observations showing a profound Ang II effect on insulin secretion *in vivo* and only a moderate effect *in vitro* (see chapter 3.1, discussion part) is still not fully understood. Further explanations could be discussed: Aside from the hypothesis of missing immune cells after islet isolation and the possible recovery of the islets after plating, one may speculate about the lack of other known factors that classically influence *in vivo* insulin secretion, including incretins or nerval innervation [268].

We tested the involvement of the incretin GLP-1 on Ang II-mediated glucose intolerance *in vivo* and could not find significant differences between the groups (Fig. 12). Unexpectedly, Ang II pretreatment even tended to increase GLP-1 secretion after an orally administered glucose bolus. This finding could explain why insulin secretion is not impaired in the Ang II treatment group in this experiment, possibly due to compensatory upregulation of GLP-1. Since the timespan of our *in vivo* experiments with the Ang II releasing pumps was much longer compared to the weekly oral administration, the GLP-1 experiments may not be comparable. To learn more about the role of GLP-1 in the Ang II-mediated effects, active GLP-1 should be measured acutely during an oral glucose tolerance test in the plasma of the mice implanted with the osmotic minipumps. One could also speculate about a possible role of other incretins such as gastric-inhibitory polypeptide (GIP), being repressed by Ang II in the *in vivo* setting.

Further research is needed to understand the underlying mechanisms of the different effects on insulin secretion by Ang II *ex vivo* versus *in vivo*.

In our study, we demonstrated strong effects on glucose metabolism and islet function *in vivo*, which are confirmed by many direct effects of Ang II on primary β-cells, the β-cell line INS-1E and pancreatic islets *in vitro*. However, one may speculate that some of the effects seen *in vivo* but not *ex vivo* could be mediated via the steroid hormone aldosterone, which is stimulated at the end of the RAS cascade [269]. There is increasing evidence that aldosterone has similar independent effects to that of Ang II or mediates the effects of Ang II [270]. Aldosterone is able to impair glucose-stimulated insulin secretion and promote insulin resistance [271], [272], even independently of the mineralocorticoid receptor [273]. The secretion of aldosterone is elevated in diabetic patients [274] and several clinical studies describe impaired glucose tolerance and insulin response in patients with hyperaldosteronism, which improved after adrenalectomy [275], [276].

Altogether, Ang II appears to cause IL-1β-mediated inflammation and β-cell disturbing effects in the context of type 2 diabetes.

5.2 Sirtuin 1

In this work, we characterized SIRT1 as essential inflammatory modulator in the pathogenesis of type 1 diabetes and autoimmune diseases. The functions of SIRT1 seem to be complex and dependent on various factors including expression level, tissue and disease context.

In the first sirtuin study, we demonstrate that modulation of SIRT1 by a single point mutation in the *Sirt1* gene influences metabolism and inflammation. Therefore, we used transduced rat INS-1E cells and a knock-in mouse model, which is carrying a SIRT1 point mutation (L107P) that was associated with familial autoimmune diseases including diabetes and colitis [215].

We show that INS-1E β-cells ectopically overexpressing the human mutated SIRT1 L107P exhibited higher susceptibility to inflammation and reduced β-cell function along with mitochondrial glucose sensing defects. These findings imply that the SIRT1 mutation causes a loss of function, which is in line with various studies reporting a beneficial role of SIRT1 overexpression in the context of inflammatory processes and insulin secretion [167], [194]. Our results suggest that the system of overexpressing human SIRT1 ectopically in INS-1E cells reflects the pathophysiological situation in the patients regarding inflammation and β-cell dysfunction.

However, the introduction of the mutation in the *Sirt1* gene in the knock-in mice, which is a less artificial model organism closer resembling the patients, revealed a new perspective to the SIRT1 mutation and its effects on immune responses and diabetes. In apparent contrast to the *in vitro* data with the transduced INS-1E cells, we observed enhanced glucose-stimulated insulin secretion in isolated islets of the heterozygous and homozygous knock-in mice compared to the control mice. Additionally, intraperitoneal glucose and insulin tolerance tests did not reveal differences between the mice, if anything, at certain ages homozygous compared to wildtype mice performed better. Given the clinical phenotype of the patients carrying the SIRT1 mutation, this finding was unexpected and led us to the hypothesis that normal function of SIRT1 may constitutively be enhanced in our homozygous mouse model, possibly leading to islet exhaustion and metabolic deterioration with

increasing age or additional triggers. Supporting this notion, nearly all key metabolic genes were basally upregulated in isolated islets from homozygous mice compared to the controls.

In line with our proposed gain-of-function theory, the knock-in mice exhibit an autoimmune-like phenotype. At a first glance this may sound contradictory since whole body and liver-specific SIRT1 knock-out mice were linked to autoimmunity [238], [249] and SIRT1 was shown to inhibit T-cell proliferation and inflammation [224], [220]. However, SIRT1 activation leads to deacetylation and degradation of the transcription factor Foxp3, which is constitutively expressed by Tregs [23], whereas blockage of SIRT1 led to improved Treg function [232].

In accordance with the theory of overactivated SIRT1 function in our knock-in mice, numbers of $Foxp3^+$ Tregs were decreased, which may lead to a reduced capacity of Tregs to maintain self-tolerance and inflammation. Expression levels of numerous genes involved in islet inflammation were induced in the mice, in agreement with the data of the INS-1E cells. In accordance with our knock-in mice, the patients carrying the mutation exhibited lower Treg levels in the blood.

Our preliminary data support the idea of not only reduced number but also impaired function of the Tregs in homozygous SIRT1 L102P mice compared to the controls. Both the number of IL-10 producing Tregs and the functional protein cytotoxic T-lymphocyte antigen 4 (CTLA-4), that is highly expressed by Tregs and that is known to decrease the inflammatory response of antigen-presenting cells, were reduced in a pilot experiment. Confirming our findings of diminished immune suppressive capacity in the homozygous knock-in mice, *ex vivo* macrophages of homozygous mice exhibited lower secretion of the anti-inflammatory cytokine IL-10 upon stimulation compared to wildtype mice in our study. Furthermore, preliminary results suggest that *ex vivo* activated T-cells from isolated lymph nodes of homozygous knock-in mice secret less IL-10 than the ones from control mice. Additionally, receptors for IL-2 and IL-22, which participate in the regulation of adaptive immune regulation and immune tolerance, were elevated in islets of homozygous mice at gene expression level. IL-2 is a cytokine that supports effector T-cell (Teff) differentiation but also the development of immune suppressive Tregs [225]. IL-22 belongs to the IL-10 family of cytokines and is classically involved in Th17-induced immune responses; however, recently it was linked to the protection from obesity-induced metabolic deteriorations [28].

The upregulation of these receptors in islets could point to a compensatory mechanism caused by the lack of IL-2 and IL-22 in homozygous mice. Since SIRT1 is able to inhibit the production of IL-2 [218], [224] and since the lack of IL-2 has been shown to play a crucial role in the development of T1D [227], our mouse model supports the hypothesis that overactivation of SIRT1 caused by the mutation may lead to decreased IL-2 and therefore promotes autoimmunity and T1D.

In contrast to our hypothesis, we observed elevated IL-2 levels in the circulation of homozygous knock-in mice compared to wildtype mice. Since IL-2 is also regulating effector T-cell development and since the suppression of IL-2-mediated activation of autoreactive T-cells has been used as immunotherapeutical treatment in T1D patients [226], it may well be that IL-2 has diverse functions in β-cells compared to other tissues and the circulation. In turn, IL-2 can also inhibit *Sirt1* transcription [218], possibly explaining the high IL-2 levels in the circulation as answer to overactivation of SIRT1. Due to the fact that low-dose IL-2 treatment can increase Treg recovery [231], the systemic increase of IL-2 in homozygous mice could also be a compensatory reaction to the low numbers of Tregs.

Our data are supported by recent studies; it has been shown that SIRT1 enhances the Th17/Treg ratio and the susceptibility to autoimmune diseases [233]. Additionally, inhibition of histone deacetylases (HDACs), the group of enzymes SIRT1 belongs to, was suggested to improve Treg function in autoimmune diseases including colitis [234], [235].

As mentioned above, there are contradictory findings about SIRT1 and autoimmunity in the literature. These discrepancies along with the divergent results of our INS-1E cell model concerning β-cell function further illustrate that SIRT1 has a variety of effects on the body, depending on the tissue and the intensity of modulation. It is not easy to create a valid model system that in all aspects reflects the situation of the patients with familial autoimmune diseases. Interestingly, the patients are carrying the SIRT1 mutation heterozygous, whereas we see the most pronounced effects in homozygous knock-in mice. Besides the one mutated allele of *Sirt1*, there are possibly other factors contributing to the human phenotype, which can not entirely be reproduced in the mice.

All of this supports the central and complex role of SIRT1 in T-cell biology and in the context of autoimmune diseases including T1D. Based on recent knowledge, inhibition of SIRT1 may improve the function and development of Tregs partly via IL-2 and Foxp3 stimulation and suppress the activity of Th17 effector cells.

One could imagine to treat the patients carrying the SIRT1 mutation with low-dose IL-2 to increase Treg recovery, as it was tested in other autoimmune conditions [231].

Based on our data, we propose that overactivation of SIRT1 may lead to an autoimmune-like phenotype, overall increase of inflammation and metabolic deterioration, possibly age-dependently or through additional triggers. To induce the precipitation of a metabolic disease in the knock-in mice, we are currently setting up a RIPova:L102P SIRT1 mouse model, resulting in mice carrying the SIRT1 mutation on an autoimmune background along with controlled induction of diabetes. It will be interesting to see if we can mimic the phenotype of the patients, including glucose intolerance and insulin resistance. Since we did not see differences in the mldSTZ model between wildtype, heterozygous and homozygous knock-in mice, it may also be interesting to activate SIRT1 through treatment with NMN or resveratrol to provoke differences in the onset of T1D. Following our hypothesis, we would expect the homozygous knock-in mice to be more prone to hyperglycemia in the mldSTZ model after treatment with additional SIRT1 activators.

In our second sirtuin study, we show that the deletion of SIRT1 specifically in β-cells negatively influenced the overall metabolic situation along with disturbances in glucose homeostasis and the induction of islet inflammation. The knock-out of SIRT1 in β-cells caused impaired glucose tolerance and lower insulin levels along with defects in mitochondrial glucose sensing and increased cytokine and chemokine production in islets. However, in the context of type 1 diabetes, SIRT1 activity in β-cells seems to be detrimental since our SIRT1 KO mice were protected from STZ-induced diabetes and the loss of body weight. These *in vivo* data of the mldSTZ experiments further support the novel way of thinking about the adverse role of SIRT1 activity in the onset of T1D.

The mice lacking SIRT1 in β-cells were protected from mldSTZ-induced hyperglycemia; in contrast to the wildtype mice they did not reach 100% diabetes induction and the body weight was less decreased throughout the experiment. The literature on the role of SIRT1 in the development of diabetes is contradictory and full of open questions. Nonetheless, it has been shown in whole body knock-out mice that lack of SIRT1 is increasing the susceptibility to STZ-induced hyperglycemia [215]. The comparison of these global SIRT1 knock-out mice with our findings in the β-cell specific SIRT1 knock-out mice conveys a subordinate role of β-

cell-derived SIRT1 in the manifestation of the demonstrated STZ-induced deteriorations. In complete agreement with our data, it was shown that mice overexpressing SIRT1 in β-cells are more prone to STZ-induced diabetes [216].

During the process of diabetes initiation, remaining islets may have a high workload to produce sufficient hormones. We hypothesize that this exhaustion may be prevented by the induction of a "β-cell rest" via knock-out of SIRT1 in β-cells, promoting longer islet survival and function along with resistance to T1D. The positive effects of β-cell rest have been shown *in vitro* and in clinical studies [259], [260]. It is suggested that β-cells with increased workload may recover when insulin secretion is inhibited or exogenous insulin is offered.

mldSTZ experiments in the context of SIRT1 may be delicate since both SIRT1 activity and mldSTZ are known to consume NAD^+, either by STZ-induced activation of PARP or because it is used as cofactor for SIRT1 [204], [136]. It is possible that the beneficial effects of the SIRT1 KO in β-cells on STZ-induced hyperglycemia arose from increased availability of NAD^+ due to the lack of SIRT1. Based on reported death cases by treatment with the SIRT1 activator SRT1720 in a mouse model of T1D [166] and based on unpublished experiments of our group with NOD mice, which are independent of NAD^+ depletion by PARP, it is more likely that SIRT1 activation itself is detrimental in the context of T1D.

Altogether, we propose that SIRT1 expressed in β-cells is a central regulator of glucose homeostasis, islet inflammation and the development of diabetes. It has diverse effects: on one hand, deletion of SIRT1 in β-cells causes impaired glucose tolerance, mitochondrial defects and elevation of inflammatory gene expression in islets. On the other hand, in the context of T1D, knock-out of SIRT1 in β-cells prevents the induction of hyperglycemia and reduced the loss of body weight.

In the last few years, the initially euphoric view of SIRT1 being a positive metabolic and inflammatory regulator has changed. It has been suggested in numerous studies that overexpression and overactivation of SIRT1 in different tissues and situations does not necessarily have beneficial effects. It was shown that strong (12.5-fold) heart-specific overexpression of SIRT1 leads to the induction of oxidative stress and cardiac dysfunction [243]. Furthermore, SIRT1 was linked to the attenuation of β-cell expansion [261], to decreased insulin sensitivity [185] and to worsening of insulin resistance in diabetic mice [252]. Overexpression of SIRT1 failed to protect mice from HFD-induced obesity [169], [172]

and impairment of insulin sensitivity [182]. Additionally, SIRT1 overexpression in rheumatoid arthritis is suggested to contribute to pro-inflammatory cytokine production and apoptosis resistance [262]. As mentioned above, the synthetic small SIRT1 activator SRT1720 that was shown to be beneficial for metabolic and mitochondrial function in animal models of T2D [164], [160], [263], [165] failed to improve metabolic conditions in other mouse studies and caused accumulated death cases, shown by Pacholec et al. [166] and by our research group in NOD mice (unpublished data).

Additionally, it has been pointed out that inhibition of histone deacetylases, the group of enzymes where SIRT1 belongs to, can improve metabolic functions, β-cell inflammation and insulin sensitivity via enhanced activity of PPARγ or glucose uptake in muscle [264], [200]. Further, the inhibition of lysine deacetylases in the NOD mouse model of T1D resulted in decreased pro-inflammatory factors in β-cells and leukocytes [265]. SIRT1 inhibition as possible treatment has been tested in other disease areas as well. Huntington`s disease (HD) is a neurodegenerative disease known to develop through transcriptional dysregulation and dysfunctional protein acetylation. The inhibition of deacetylation by SIRT1 blockage was well tolerated in a human study [266] and resulted in improvement of HD pathology in several cell and mouse models [267].

All of these findings suggest a negative role of SIRT1 overactivation in the context of diabetes. Thus, inhibition of SIRT1 as histone and protein deacetylase may have beneficial effects in the development of T1D.

To interpret the data of our both sirtuin 1 studies, we revealed new perspectives for understanding the complex functions of SIRT1 as an essential player in immune responses and type 1 diabetes. Based on our data of the knock-in mice SIRT1 L102P, we hypothesize that SIRT1 overactivation leads to an altered immunological balance with increased inflammation, improved islet function and glucose tolerance. This may lead to exhaustion of the β-cells and may accelerate the development of type 1 diabetes, as we saw protective effects through β-cell specific SIRT1 knock-out. Accordingly, inhibition and not activation of SIRT1 in the context of type 1 diabetes could have beneficial effects. Nonetheless, targeting SIRT1 is a delicate issue and seems to depend on various factors. There is evidence that the functions of SIRT1 are required in physiological states and for adaptation to stress

conditions. However, in certain inflammatory pathologies, such as the onset of T1D, it may have therapeutical value to inhibit and not enhance SIRT1 activity.

Altogether, this study revealed the influence of two important inflammatory modulators, sirtuin 1 and angiotensin II, on the development of diabetes. Several factors have been implicated with islet inflammation and β-cell damage, including glucose, free fatty acids, amyloid and also β-cell antigens. However, a variety of additional molecules may contribute to the deterioration of glucose homeostasis by modulating inflammatory processes. Based on our data, we propose that inhibition of angiotensin II in the context of type 2 diabetes and lowering of sirtuin 1 activity in the development of type 1 diabetes may have therapeutical value.

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